Journal of Immunological Methods 415 (2014) 31–35

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

Raman spectroscopic modeling of early versus late T-lymphocyte activation via differential spectral detection of receptor expression

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ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form 7 October 2014
Accepted 13 October 2014
Available online 20 October 2014

Keywords:
Acute rejection
Raman Spectroscopy
Renal transplantation
CD69 receptor expression
CD25 receptor expression
CD71 receptor expression

ABSTRACT

The proven efficacy of renal transplantation has made it the definitive treatment for end-stage renal disease. Despite its wide acceptance, transplantation has been limited by organ shortages. In the face of this, preservation of allograft longevity is essential. The predominately T cell-driven process of acute rejection (AR) can lead to graft dysfunction and even graft loss. As a marker for AR screening, serum creatinine has a low sensitivity and specificity. This has warranted the development of more accurate screening/diagnostic tools such as Raman Spectroscopy (RS) which has been demonstrated in previous studies to accurately quantify T cell activation. In this study we further explore its application by modeling the dynamic process of cell surface receptor expression during T cell activation. 50 mitogen (Concanavalin A and pokeweed) activated T cells were stained with CD69, CD25, and CD71 monoclonal antibodies (mAbs) at 48 and 72 hour time points. In parallel, 50 activated T cells were analyzed using RS at these same time periods. At 48 h there was high expression of the CD69 cell surface receptor detected via mAb staining with no appreciable binding of CD25/CD71 fluorescent tag. In conjunction, 48 hour RS-analyzed T cells demonstrated a significant peak difference at the 1585 cm^{-1} position which represented a 63% ($p = 0.01$) increase in peak magnitude when compared with the 72 hour samples. By contrast, the 72 hour data demonstrated an attenuation of the CD69 expression and increased CD25/CD71 expression. The corresponding RS analysis showed two significant peak differences at the 903 cm^{-1} and 1449 cm^{-1} positions that were not present at 48 h. These differences in Raman shifts resulted in a 40% ($p = 0.04$) and a 59% ($p = 0.001$) increase in peak magnitudes at these positions, respectively. This study serves to further validate RS as a screening modality capable of not only detecting T cells early in the activation process through the spectral signatures associated with CD69, but also quantifying the persistent expression of CD25 and CD71. This provides a foundation for the development of a system capable of the accurate assessment of acute and maintenance immunosuppression efficacy at the molecular level.

1. Introduction

The proven efficacy of renal transplantation has made it the definitive treatment for end-stage renal disease (ESRD). However, despite its wide acceptance, it has been limited by the continued shortage of available organs (Anon.). It is therefore imperative that the functionality and survival of

Abbreviations: ESRD, end-stage renal disease; AR, acute rejection; RS, Raman Spectroscopy; SCE, serum creatinine elevation; mAb, monoclonal antibody.

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http://dx.doi.org/10.1016/j.jim.2014.10.001
0022-1759/

each graft be maximized. One condition that negatively impacts graft outcomes is the T lymphocyte-driven process of acute rejection (AR). Although AR is a reversible process in the vast majority of cases when detected early, repeated episodes and delayed diagnosis can lead to sequelae that jeopardize long-term graft function and survival (Cosio et al., 1997; Tesi et al., 1993). Despite the widespread use of serum creatinine elevation post-transplant as a marker for AR, it represents a nonspecific, nonsensitive finding that becomes apparent only after significant histologic damage to the transplanted organ has already occurred (Gaber et al., 1996; Nankivell et al., 2004).

In response to the limitations of the current AR diagnostic algorithm, many centers have searched for alternative methods of non-invasive detection of AR. One of these methodologies is Raman Spectroscopy (RS), a low-cost, monochromatic laser-based technology that has shown high sensitivity in detecting alloreactive T lymphocytes involved in the AR process (Brown et al., 2009a). Moreover, RS has demonstrated high specificity in spectrally differentiating among various other causes of T lymphocyte activation (Brown et al., 2009b). However, in order to fully model AR using RS, we must be able to not only detect alloreactive cells, but also chronicle the differential *timing* of T cell surface receptor expression at the molecular level during critical steps of the process.

The CD69 receptor, a 22.5 kD type II transmembrane homodimeric glycoprotein, is transiently expressed as early as 2 h after activation and is subsequently down-regulated by 55 h post-activation (Simms and Ellis, 1996; Posselt et al., 2003; Mardiney et al., 1996; Alonso-Camino et al., 2009). This represents the "early" phase of activation (Janeway et al., 1999). By contrast, the CD25 receptor, a 55 kD low-affinity IL-2 receptor α chain type I transmembrane glycoprotein, and the CD71 homodimeric type II transmembrane human transferrin receptor follow a delayed expression pattern, appearing at 13, 25, and 48 h following activation, respectively (Maino et al., 1995; Taniguchi and Minami, 1993). These receptors remain at high levels throughout the activation/clonal expansion process and represent the "late" phase of activation. Therefore the purpose of this present study was to utilize RS to demonstrate the spectral evolution of CD69, CD25, and CD71 cell surface receptor expression during the T lymphocyte activation process.

2. Materials and methods

2.1. Cell preparation and mitogen induction

Prior approval for the study was obtained from the Wayne State University Institutional Review Board. Sodium-heparinized venous blood was collected from healthy participants and incubated with 40 μl of a 1/3 mixture of Concanavalin A (*Canavalia ensiformis* agarose; Sigma, St. Louis, MO) and pokeweed (*Phytolacca americana* lectin; Sigma, St. Louis, MO). Following a 24-hour mitogen incubation period, T lymphocytes were isolated directly from the peripheral blood using a Dynal T cell negative isolation kit (Invitrogen, Canoga Park, CA) and resuspended in complete media. Activated T lymphocytes were confirmed and isolated using flow cytometry. This purely activated (and antibody free) T cell population was then cultured at 37 °C in 5% CO_2 in multi-well plates in complete media containing recombinant interleukin 2 (50 U/ml; Becton Dickinson, San Jose, CA). Negative control samples were cultured in parallel and consisted of T lymphocytes pretreated with Mitomycin C (Sigma, St. Louis, MO) (0.5 mg/ml added to each 1 ml of cell suspension) for 20 min. T lymphocyte cultures were continued for 72 h with daily viability testing, daily RS analysis, and monoclonal antibody (mAb) staining at 48 and 72 h.

2.2. mAb staining

Representative aliquots of mitogen-activated T lymphocytes were collected at 48 and 72 h following initial stimulus and washed three times with PBS. All cells collected at each time period were stained with a 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Rockford, IL) nuclear stain that was contained in an anti-fade solution. In addition, cells were divided into two groups at each time interval. The first group was treated with paraformaldehyde (Sigma, St. Louis, MO) and incubated overnight with the cell surface mAb, CD69 (Becton Dickinson, San Jose, CA). The second group was treated with a permeabilizing solution (Becton Dickinson, San Jose, CA) and incubated overnight with an intracellular antibody cocktail containing a CD25/CD71 (Becton Dickinson, San Jose, CA) mixture with subsequent fixing in paraformaldehyde. Cells were viewed using a Nikon Eclipse TE 2000-U inverted microscope with images being captured and processed using Metavue 6.2r5 software (Downingtown, PA).

2.3. RS analysis

RS analysis was carried out daily on non-antibody bound lymphocytes. Spectra from the 48- and 72-hour time points were compared with mAb staining. Prior to analysis, all samples were washed with PBS. Cells were allowed to settle within the analysis reservoir placed on a leveled microscope stage. Only rounded, non-adhered, isolated, and individualized T lymphocytes were selected for the study. Each sample was analyzed using an identical protocol and spectroscopic parameters. Using an enhanced video imaging system, T lymphocytes were aligned in the laser target area so that the focus point was centered on the lymphocyte. Images were captured before and after each measurement, with no noted cell shifting or destruction observed. Wire 2 software (Renishaw plc, Old Town, United Kingdom) was utilized in conjunction with the Raman system for quantitative measurements. RS measurements were conducted using a Renishaw InVia 2000 microscope–spectrometer with a Leica 63× water immersion objective (NA = 1.20). T lymphocytes were analyzed using a 514.5 nm (green) Ar^+ excitation wavelength. Laser power was set at 50% (8–12 mW) for a 2–4 μm laser spot size. Spectra were collected in the backscattering geometry with a 10 second integration time over a range of 500 to 1700 cm^{-1}. The spectral resolution was ~4 cm^{-1}.

2.4. Statistical analysis

Prior to statistical analysis, all data were checked for various types of noise. Fluorescence was subtracted using a modified cubic-spline algorithm that required no a priori knowledge of the spectra. In addition, a median filter was applied to raw data to eliminate any cosmic ray or spikes in the data. The Raman spectra from all data contained 1184 data points. To determine