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Raman Spectroscopic Differentiation of Activated versus Non-activated T lymphocytes: An *in vitro* study of an acute allograft rejection model¹

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INTRODUCTION

Recent advances in immunosuppressive therapy, medical management, and surgical technique have further improved patient and graft outcomes in renal transplantation, thereby expanding the candidate pool to include higher-risk recipients (1). Even with use of more marginal deceased-donor organs and pushing the use of live-donor organs to the limit, however, the disparity between supply and demand for organs continues to grow exponentially (2). In the face of a profound organ shortage, it becomes paramount that the longevity of existing grafts be maximized. One condition that negatively impacts this longevity is acute rejection (AR). AR occurs in approximately 15–20% of patients during the first year posttransplant and remains an important factor leading to decreased long-term graft function and survival (3,4). Reliable screening for AR has proven to be problematic. Serum creatinine, the most commonly used indicator of transplant function (5), is not an accurate reflection of glomerular filtration rate, lacks sensitivity and specificity (6), and becomes elevated only after significant histologic damage has occurred (7). Based on the aforementioned observations, a rapid, noninvasive, highly specific and sensitive screening test for AR is warranted.

It has been well established that T lymphocytes are the predominant immune cells involved in the process of AR (8,9). The hallmarks of T lymphocyte activation are an increase in cytotoxic specific DNA transcription, immuno-specific proteins (mainly perforin and granzymes) and a characteristic up-regulation of cell surface receptors, notably CD69, CD25, CD71, and CD3 (10–13). We hypothesize that the differentiation of activated and non-activated T lymphocytes, in blood or urine, can be accomplished by using a biomedical tool that analyzes these cell surface receptors.

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Raman spectroscopy has been used for years in industry and engineering to characterize and identify materials based upon their energy modes that are unique to that particular substance. Recently, this technique has been extensively applied in the biomedical arena for the detection of a variety of cancers and even pathogens present in the environment (14–16). Raman spectroscopy utilizes a laser source which interacts with various vibrational and other modes leading to light scattering of the elastic and inelastic nature. The energy spectrum of the inelastic scattered light is directly related to vibrational, rotational and other low energy modes specific to the biochemical composition of the substance under investigation. These energy modes serve as a molecular fingerprint and can be rapidly translated into a unique spectral signature using minute amounts of sample. To our knowledge, this is the first study utilizing Raman spectroscopy to analyze the differences in receptor content found on the cell surface of intact activated and non-activated T lymphocytes.

MATERIALS AND METHODS

Cell Preparation

Following approval from the Wayne State University Human Investigation Committee, mononuclear cells were obtained using freshly drawn (within 3 hours of experimentation) sodium-heparinized venous blood collected from healthy individuals and separated according to density via high molecular weight sucrose polymer (ficoll) as described by Boyum (17). The resultant cells were washed three times using Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) and suspended in McCoy's solution/5% fetal calf serum (Biofluids, Rockville, MD) prior to T cell isolation. Following mixed lymphocyte reaction, B lymphocytes were removed by negative selection using FluoroBeads-B immunomagnetic beads and magnetic sorter (One Lambda, Canoga Park, CA). This process was repeated twice with chilled PBS washings used to suspend cells between magnetic separations. Cell viability was >98% as checked by vital dye exclusion. T lymphocyte enriched samples were suspended in complete medium and aliquoted for subsequent experimentation.

Peripheral Blood T Lymphocyte Samples

Three distinct T cell sample groups were created for the study, consisting of activated, inactivated, and resting T lymphocytes. The activated T lymphocyte samples were the product of a two-way mixed lymphocyte culture (MLC) as described by Dupont and colleagues (18). The inactivated group was created using T lymphocyte/stimulator samples from two unrelated individuals that were pretreated for 20 minutes with Mitomycin C (0.025 mg of Mitomycin C (0.5 mg/ml) to each 1 ml of cell suspension) at 37°C and washed twice using HBSS prior to start of MLCs. Efficacy of Mitomycin C was verified using double inactivation control cultures which were run in parallel. Finally, the resting T lymphocyte sample group was defined as T cells obtained by methods described previously, which were neither stimulated via MLC nor inhibited by Mitomycin C treatment. Antigen presenting cells (APCs) were subsequently removed from activated, inactivated, and resting T cell cultures via steel wool column prior to Raman analysis. All cultures were incubated at 37°C in 5% CO₂ in parallel over a 7-day period with Raman spectroscopy, antibody analysis, and viability assays carried out daily.

Independent Verification of Activation/Inactivation via Antibody Staining

Activation and inactivation of representative aliquots were verified using FastImmune anti-CD69 antibody (Becton Dickinson, San Jose, CA), with 94% of MLC (activated) and 100% of Mitomycin C-treated (inactivated) T lymphocytes staining positive and negative, respectively, using protocols described elsewhere (19). In addition, there was no detected activation due solely to physical handling of specimens. Stained 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).

Raman Spectroscopy

Prior to analysis, all samples were washed with PBS (Invitrogen, Carlsbad, CA). Cells were allowed to settle within the analysis reservoir placed on a leveled microscope stage. Only rounded, non-adhered, isolated and individualized T cells were selected for study. Each sample was analyzed using identical protocol and spectroscopic parameters. Using an enhanced video imaging system, T cells 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 measurement.

Raman spectroscopic measurements were conducted using a Renishaw InVia 2000 microscope-spectrometer with a Leica 63X water immersion objective (NA= 1.20). Due to the complexity of biological structures which can embody numerous vibrational modes, two different excitation wavelengths were used in this study. T lymphocytes were analyzed using both 785 nm (red) diode and 514.5 nm (green) Ar⁺ excitation wavelengths. Laser power was set at 50% (8–12 mW) for a 2–4 μ m laser spot size. Spectra were collected in the backscattering geometry with a 10 second integration time over a range of 600 to 1700 cm^{-1} . The spectral resolution was $\sim 4 \text{ cm}^{-1}$. A schematic of the Raman spectroscopic system is depicted in Figure 1.

Statistical Analysis

Prior to statistical analysis, all data was checked for various types of noise. There was no significant fluorescence observed for the 785 nm wavelength excitation. However, with the 514.5 nm excitation, Raman spectral data showed fluorescence uniformly increasing over the entire range of Raman shift. This fluorescence was subtracted out 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 the independent variations in the intensity which basically represents different pathological states in the sample, we used principal component analysis to reduce dimensionality in the data. We found that seven eigenvectors were able to capture more than 97% of total variance in the data. Student's t-test was utilized to further quantify differences in peak ratios. The data were further analyzed for the presence of various independent groups using discriminant function analysis (DFA) with the aforementioned eigenvectors as input variables. Graphical and quantitative analysis of data was accomplished using SPSS version 15 software (Statistical Software, Chicago, IL).

RESULTS

Raman Spectroscopy Results - Red (785 nm) Laser

A total of 23 activated, 12 inactivated, and 20 resting T lymphocytes were examined. A representative Raman spectrum of resting T lymphocytes is depicted in Figure 2 with proposed peak assignments for 1182 cm^{-1} and 1195 cm^{-1} positions shown in Table 1. There was no observed cellular disruption during measurements, and after comparison of activated and non-activated (resting and inactivated) spectra from 600–1700 cm^{-1} , qualitative differences were seen at the 1182 cm^{-1} and 1195 cm^{-1} positions.

Representative activated and inactivated T lymphocyte spectra are depicted in Figures 3A and 3B, respectively. Ratios of the 1182 cm^{-1} and 1195 cm^{-1} peaks were determined by comparison of amplitudes with respect to a designated baseline depicted by a dashed line in the figures. The average peak ratios for the 1182 cm^{-1} and 1195 cm^{-1} positions for the activated, inactivated, and resting T lymphocyte groups were 0.98 ± 0.04 , 1.13 ± 0.10 , and 1.10 ± 0.05 , respectively. Significant differences in peak ratios existed between activated and resting as

well as between activated and inactivated T lymphocyte groups ($p = 0.001$ and $p = 0.0001$, respectively). There was a trend observed in the differences between inactivated and resting T lymphocytes ($p = 0.07$).

A summary of all Raman spectral data collected with 785 nm excitation for activated, inactivated, and resting T lymphocytes is shown in Figure 4. Based upon the distribution of raw data, 3 distinct sectors were assigned for grouping purposes: Sector I, inactivated T cells; Sector II, activated T cells; Sector III, resting T cells. 95.7% of activated T lymphocytes clustered in Sector II, with the remaining 4.3% clustering in Sector I. One hundred percent of inactivated T lymphocyte samples clustered in Sector I, while 60% of the resting T lymphocytes were found in Sector III, and 40% in Sector I.

Raman Spectroscopy Results - Green (514.5 nm) Laser

A total of 28 activated, 16 inactivated, and 15 resting T lymphocytes were examined. A representative Raman spectrum of resting T lymphocytes is depicted in Figure 5. There was no observed cellular disruption following laser exposure.

The average peak ratios for the 1182 cm^{-1} and 1195 cm^{-1} positions for the activated, inactivated, and resting T lymphocyte groups were 0.93 ± 0.05 , 1.15 ± 0.10 , and 1.09 ± 0.09 , respectively. Representative activated and inactivated T lymphocyte spectra are depicted in Figures 6A and 6B, respectively. Significant differences in peak ratios existed between activated and inactivated as well as between activated and resting T lymphocytes ($p = 0.001$ and $p = 0.006$, respectively). In contrast, there was only a trend seen in differences between inactivated and resting T lymphocytes ($p = 0.08$).

Figure 7 demonstrates the DFA plot for the Raman spectral data collected with 514.5 nm excitation source for the activated, resting, and inactivated T lymphocytes. 89.3% of the activated T lymphocytes grouped into Sector II, with 3.6% falling into Sector I and 7.1% clustering into Sector III. 93.8% of the inactivated T lymphocytes were found in Sector I, while 6.2% marginalized into Sector III. 86.6% of the resting T lymphocytes were found in Sector III with 13.4% clustering into Sector I.

DISCUSSION

The purpose of this study was to utilize Raman spectroscopy to establish biomedical-based differences between activated and non-activated T lymphocytes which can serve as the basis of a non-invasive screening modality for AR. Although other noninvasive biomedical technologies to detect AR have been proposed, many require disruption of cellular biology and immunological integrity followed by labor intensive processing (20,21), while others are limited by the need to analyze the up-regulated byproducts of an intact immune system (22, 23). These byproducts, in turn, may be influenced by other endogenous and exogenous factors, making direct correlation with AR difficult. In contrast, we have approached the study of AR directly by studying the activation patterns via the receptors of the T lymphocyte, the principal effector cell of AR (8,9).

Initial qualitative comparison of activated and non-activated T lymphocytes yielded differences at four distinct positions in the Raman spectrum: 788 cm^{-1} ; 1000.43 cm^{-1} ; 1182 cm^{-1} ; and 1195 cm^{-1} . Peaks at 788 cm^{-1} and 1000.43 cm^{-1} have been previously identified and described as markers for cellular viability (24). However, we observed a very distinct spectral difference in peak positions 1182 cm^{-1} and 1195 cm^{-1} . These differences, and more importantly their ratio to one another, have not been previously described. Although the exact peak assignments for the 1182 cm^{-1} and 1195 cm^{-1} positions remain cryptic, our results suggest that the changes observed, whether they be in DNA/RNA, carbohydrate, lipid or

protein, are due to activation. This process of activation spans from initiation of the genetic apparatus via immuno-specific transcription factors to the up-regulation of glycoproteins on the T cell surface. Indeed, this process encompasses all peak assignment categories and importantly, can be quantified by our Raman spectroscopic system.

The *in vivo* immunologic landscape concerning the activation of T lymphocytes exists on a continuum, with the breadth of the spectrum ranging from anergy to full activation. The observation that a small subset of activated T lymphocytes in both the 785 nm and 514.5 nm cohorts were found in Sectors I and III, or the inactivated and resting T lymphocyte sectors, respectively, can be explained by the concept of anergy, here defined as the inability to up-regulate receptors of activation. To further address and verify this issue, studies are underway using T cell stimulation protocols involving super-antigens that are independent of MHC restriction. This should yield a more uniform distribution of T lymphocytes into Sector II, and truly test this concept of anergy.

No inactivated T lymphocytes were found in Sector II. This important observation verifies the notion that although we would expect a small percentage of lymphocytes to fail to activate due to anergy, we would not expect a Mitomycin C-treated T cell to activate and thus falsely group with activated samples in Sector II. This suggests that a screening modality with high specificity could be achieved with this technology.

Although the difference in average peak ratios between resting and inactivated T lymphocyte groups of both red and green lasers did not quite reach statistical significance, their DFA plots were graphically different, allowing for discrimination. The exchangeability found between Sectors I and III that did not exist for Sector II suggests that there is a threshold of activation required to populate the T cell surface with a density of receptors necessary to create a distinct activation signature.

Based upon Raman spectra from excitation wavelengths 785 nm and 514.5 nm, average peak ratios, and DFA plots, there was a significant difference observed between the activated T lymphocyte group and the non-activated (resting and inactivated) T lymphocyte groups. When considering both wavelengths, the red laser demonstrated a tighter association of T lymphocytes to their designated sector, with 96% of activated T lymphocytes aggregating into Sector II and 100% of inactivated T lymphocyte samples aggregating into Sector I. We will utilize this observation to guide future experiments in which we will employ the 785 nm (red) laser exclusively.

To ascertain the validity and feasibility of this Raman-based methodology, it was necessary to begin our studies using blood samples in order to establish signatures of activation and non-activation. These foundational signatures have informed further preliminary studies which are underway utilizing urine, which is the direct bio-run off from the transplanted graft and thus should provide a more accurate reflection of the immunologic environment of the renal allograft. In addition, a number of alternate foci of activation/non-activation differences are currently under investigation with the goal of grouping several separate signatures to create a comprehensive panel that will have the increased specificity and sensitivity required for an effective screening tool.

CONCLUSION

Raman spectroscopy can detect significant differences between activated and non-activated T lymphocytes and thus establish unique signatures for both. These signatures form the basis of a detection system which may facilitate the development of a specific and rapid screening tool for AR of renal transplants.

Abbreviations used

AR	Acute Rejection
MLC	Mixed Lymphocyte Culture
ATC	Activated T Cell
ITC	Inactivated T Cell
RTC	Resting T Cell
MHC	Major Histocompatibility Complex

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Figure 1. General schematic for Raman spectroscopic analyzer with representative spectra. 1-laser; 2-detector; 3-charge-coupled device; 4-diffraction grating; 5-slit; 6-Raman scattered light; 7-Rayleigh filters; 8-scattered light; 9-sample with lymphocyte culture.

Figure 2. Raman spectroscopic analysis of T lymphocyte using 785 nm laser with range of 600–1700 cm^{-1} . Representative T cell (Inset a) demonstrating cellular integrity following analysis. (*) Indicates area of focus containing the 1182 and 1195 cm^{-1} peaks.

Figure 3.

(A) Raman spectroscopic analysis of 1182 cm⁻¹ (*) and 1195 cm⁻¹ (^) peaks of activated T lymphocyte for the 785 nm wavelength. Inset a shows independent verification of activation via anti-IL-2/CD69/CD4/CD3 antibody staining. (B) Spectroscopic analysis of inactivated T lymphocyte with inset b demonstrating non-activation. Ratios determined by amplitude with respect to designated baseline (dashed line).

Figure 4.

Discriminant function analysis of T lymphocytes at 785 nm wavelength plotted without (A) and with (B) assigned sectors. Sectors I, II, and III correspond to inactivated, activated, and resting samples, respectively.

Figure 5. Raman spectroscopic analysis of T lymphocyte using 514.5 nm laser with range of 600–1700 cm^{-1} . Representative T cell (inset a) demonstrating cellular integrity following analysis. (*) Indicates area of focus containing the 1182 and 1195 cm^{-1} peaks.

Figure 6.

(A) Raman spectroscopic analysis of 1182 cm^{-1} (*) and 1195 cm^{-1} (^) peaks of activated T lymphocyte for the 514.5 nm wavelength. Inset a shows independent verification of activation via anti-IL-2/CD69/CD4/CD3 antibody staining. (B) Spectroscopic analysis of inactivated T lymphocyte, with inset b showing non-activation. Ratios determined by amplitude with respect to designated baseline (dashed line).

Figure 7. Discriminant function analysis of T lymphocytes at 514.5 nm wavelength plotted without (A) and with (B) assigned sectors. Sectors I, II, and III correspond to inactivated, activated, and resting samples, respectively.

Table 1

Raman frequencies of measured range and their peak assignments.

Lymphocyte Raman Shift (cm-1)	Assignments
1583	Nucleotide (Adenine(A) + guanine(G))
1319	G, Protein: Carbon (C)-Hydrogen (H) deformation
1208	A, Thymine (T), protein: amide III
1195 – 1182 *	Tyrosine, Phenylalanine (P), Protein: C–H bend A,T, protein: amide III
1175	Tyrosine, P, Protein: C–H bend
1155	Protein, C-C, C-Nitrogen (N) stretch
1126	Protein: C-N stretch
1095	DNA phosphate (PO ₂ ⁻)
1053	C-Oxygen (O) stretch, protein: C-N stretch
1031	P, protein: C–N stretch
1005	P vibration (C-C) symmetric
788	Uracil-T, C, DNA (O-PO ₂ ⁻ -O backbone stretching)
746	T- breathing mode
725	A – breathing mode
666	G,T, Tyrosine, G backbone in RNA
640	C-5 stretch C-C twist Tyrosine
618	C-C twist

* Proposed peak assignments (25–28)