

Research paper

Activation-dependent phases of T cells distinguished by use of optical tweezers and near infrared Raman spectroscopy

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Abstract

Near-infrared Raman spectroscopy may provide a highly sensitive, noninvasive means to identify activation status of leukocytes. The purpose of the current study was to establish Raman spectroscopic characteristics of T cell activation. Activation of the RsL.11 T cell clone in vitro with Con A resulted in specific decrements in band intensities at 785, 1048, 1093, and 1376 cm^{-1} but did not alter a majority of other band intensities including those at 1004 cm^{-1} (phenylalanine) and 1660 cm^{-1} (amide bonds). Activation-dependent decrements in these band intensities occurred subsequent to IL-2 production and correlated closely with T cell blastogenesis. Activation-dependent decrements in these band intensities were not strictly a function of cell size because the same observations were noted in size-controlled comparisons of resting and activated T cells. Like the RsL.11 clone, freshly isolated thymocytes that were activated by Con A or IL-2 showed decrements in particular emissions. These findings indicate that near-infrared Raman spectroscopy can be used as a noninvasive technique to reveal the activation status of single living T cells.

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1. Introduction

The use of optical tweezers coupled with Raman spectroscopy for single-cell analysis is an emerging

technology (Xie et al., 2002, 2003; Xie and Li, 2003) that may play an important role in revealing the functional status of small numbers of leukocytes isolated from biological samples. Optical tweezers allow capture and immobilization of individual leukocytes in a liquid medium by use of a tightly focused near-infrared laser beam. Raman spectroscopy excited with the same laser beam can generate a highly characterized “fingerprint” for each substance inside the cell by measuring the unique

Abbreviations: CM, Conditioned medium—complete RPMI medium supplemented with recombinant rat IL-2.

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vibrations of particular molecules. Therefore, Raman spectroscopy may provide a sensitive indicator of activation-dependent changes in protein and nucleic acid metabolism and may reflect generation of reactive oxygen and nitrogen intermediates. Lymphocyte activation causes global increases in nucleic acid and protein biosynthesis and alters pH, redox potential, and ionic constituency of the intracellular environment. Activation-dependent changes in the intracellular environment would predictably affect the dominant Raman spectra of intracellular macromolecules.

The current study focuses on the combined use of optical tweezers and Raman spectroscopy to analyze T cell activation. The data indicate that Raman spectroscopy provides a useful measure of T cell activation in that rested and activated T cells from the same clone can be readily distinguished by specific Raman band intensities. Overall, this information may provide a new window on the analysis of T cells isolated from biopsies or other biological materials and may provide insight into whether resident T cells represent resting bystander T cells or activated, antigen-ligated T cells.

2. Materials and methods

2.1. Animals and reagents

Lewis rats (Harlan-Sprague Dawley, Indianapolis, IN) were maintained at East Carolina University School of Medicine. Animal experimentation was done in accordance with protocols approved by the Institutional Animal Care and Use Committee. Con A (Sigma) was used at a final concentration of 2.5 $\mu\text{g}/\text{ml}$. Recombinant rat IL-2 was derived from a baculovirus expression system (Norris et al., 2001; Mannie et al., 2003).

2.2. Lewis rat T cell lines and cell culture conditions

The Lewis rat CD4⁺ RsL.11 clone is specific for myelin basic protein and I-A¹ MHC class II glycoproteins (Mannie and Norris, 2001; Patel et al., 2001). T cells were propagated in complete RPMI 1640 medium supplemented with IL-2 (CM). Complete RPMI medium consisted of 10% heat-

inactivated fetal bovine serum (Summit, Boulder, CO), 2 mM glutamine, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin (Whittaker Bioproducts, Walkersville, MD), and 50 μM 2-ME (Sigma) (cRPMI). CM contained 0.4% v/v supernatant of a Sf 9 insect cell culture infected with a recombinant rat IL-2 baculovirus.

2.3. Time-course of T cell activation

RsL.11 T cells ($10^5/\text{well}$) were cultured in complete RPMI without a stimulus, with 2.5 $\mu\text{g}/\text{ml}$ Con A, or with the combination of IL-2 and Con A for designated durations. At designated intervals, cells were used for Raman spectroscopy, and supernatants were transferred into replicate plates to measure IL-2 bioactivity. CTLL cells ($2.5 \times 10^4/50 \mu\text{l}$ cRPMI/well) were used as an indicator cell line for IL-2 bioactivity. CTLL cells were cultured with 100 μl of supernatant for 48 h, and 10 μl of a MTS/PMS solution [2.9 mg/ml MTS (Promega) and 0.1 mg/ml PMS (Sigma)] was added to each well. Plates were read the next day at 492 nm on an Anthos ELISA Reader (ACCSales, Chapel Hill, NC). Mitogen-induced IL-2 production was measured as the mean OD values from stimulated cultures minus mean OD values from control non-stimulated cultures. Assays were routinely performed in triplicate cultures per group.

2.4. Activation of RsL.11 T cells and thymocytes

RsL.11 T cells ($5 \times 10^5/\text{ml}$) were cultured for 3 days with IL-2 in the presence or absence of irradiated splenocytes ($5 \times 10^6/\text{ml}$; 3000 rads of γ -irradiation) and Con A as designated. Thymocytes ($10^7/\text{ml}$) were cultured for 3 days in complete RPMI without a stimulus, with IL-2, or with the combination of IL-2 and Con A. After this 3-day culture, cells were analyzed by Raman spectroscopy.

2.5. Analysis of individual T cells by use of optical tweezers and near-infrared Raman spectroscopy

The experimental setup of the optical tweezers and Raman spectroscopy system was described in detail previously (Xie and Li, 2003; Xie et al., 2003). A circularized near-infrared beam from a low-

power laser diode at near 785-nm was introduced in an inverted microscope (Nikon TE-2000S) equipped with an objective (100 \times , NA=1.30) to form a single-beam optical trap. The wavelength of the diode laser was temperature-stabilized to avoid drifts. A cell in a liquid medium was selectively trapped with the radiation force yielded by the focused laser beam. The same laser excited Raman scattering from the trapped cell. The backscattered light was collimated with the same objective lens and passed through a 100- μ m confocal pinhole aperture to reject most of the off-focusing Rayleigh scattering light. Two interference notch filters were used to remove most of the on-focusing Rayleigh scattering light. The Raman scattering light was then focused onto the entrance slit of an imaging spectrograph equipped with a liquid-nitrogen-cooled charged-coupled detector (CCD). The image of the trapped cell was observed with an illumination lamp and a video camera system. The spectral resolution of the system was estimated to be ~ 6 cm^{-1} . In order to sample and

average most of the trapped cell, the laser beam was steered rapidly (~ 200 Hz) by a pair of computer-controlled Galvo mirrors across the major area of the cell. Because the steering speed was very fast, the cell was found not to follow the steering beam so that the major portion of the cell was excited within the acquisition time. The acquisition time was typically 120 s for an individual cell with an excitation power of 15 mW at 785 nm.

3. Results and discussion

T cell activation involves substantial alterations in the intracellular environment of proteins and nucleic acids and therefore may cause specific alterations in band intensities detected by near-infrared Raman spectroscopy analysis of laser-trapped living cells. To test this hypothesis, the RsL.11 clone of CD4⁺ T cells was cultured with no stimulus (Fig. 1A), Con A (Fig. 1B), or the combination of Con A and IL-2

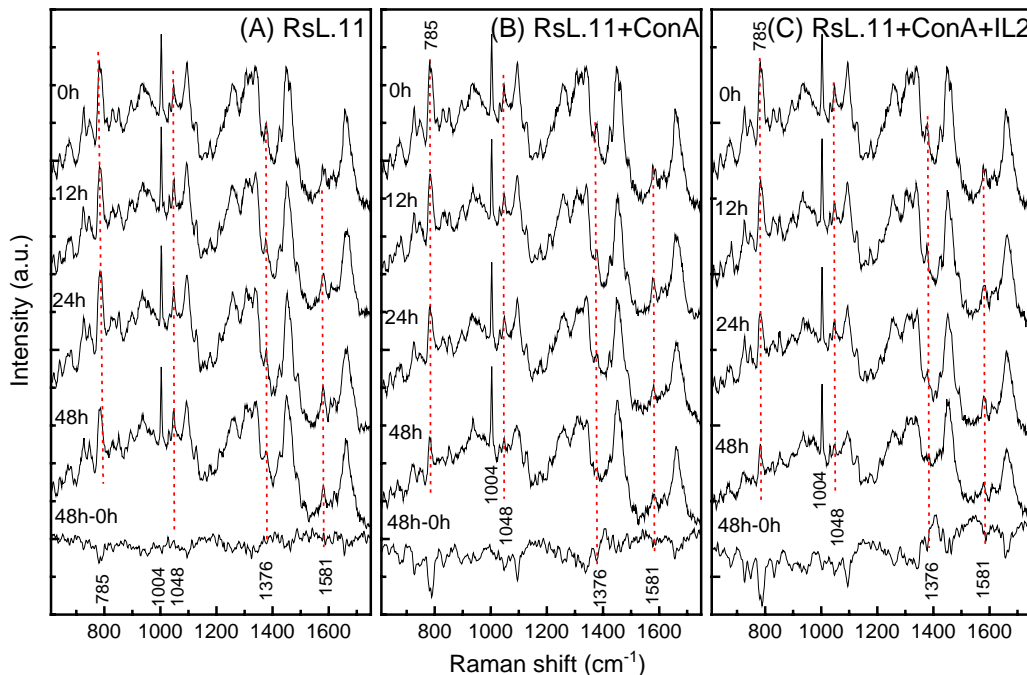


Fig. 1. Unique Raman spectroscopic profiles distinguish rested and activated CD4⁺ T cells. RsL.11 T cell were cultured with (A) no stimulus, (B) Con A, or (C) the combination of Con A and IL-2. RsL.11 T cells were obtained at the onset of culture (0 h), or were harvested at 12 h, 24 h, or 48 h for analysis by near-infrared Raman spectroscopy. Difference spectra (bottom trace) were obtained by subtracting the spectra from the 0-h time-point from the spectra for the 48-h time-point. Each spectrum was the average of 10 cells.

(Fig. 1C) for designated durations. After 24 or 48 h of culture (Fig. 1B–C), activated T cells exhibited decrements in band intensities at resonance frequencies of 785, 1048, and 1376 cm^{-1} whereas other band intensities were unaffected including those at 1004 cm^{-1} (phenylalanine) and 1660 cm^{-1} (amide bonds). In contrast, rested T cells cultured without mitogen did not exhibit altered band intensities. Activation-dependent decrements in band intensities at 1048 and 785 cm^{-1} were late events in the activation cascade that correlated closely with blastogenesis (Fig. 2, compare Fig. 2A and B with Fig. 2D). Con A-stimulated production of IL-2 was detected at 6 hrs and was nearly maximal at 12 hrs

of culture (Fig. 2C) whereas cell enlargement and decrements in band intensities at 1048 and 785 cm^{-1} were initially evident at 24 hrs and were maximal by 48 hrs of culture. Activation-dependent decreases in the magnitude of the 1376 cm^{-1} band were also a late event in the activation cascade (Fig. 1). These data indicate that near-infrared Raman spectroscopy can be used as a noninvasive means to reveal the activation status of individual CD4^+ T cells. The tentative assignment of particular frequencies in the Raman profile to particular chemical moieties is provided in Table 1.

Although time-course experiments revealed a correlation between the onset of blastogenesis and

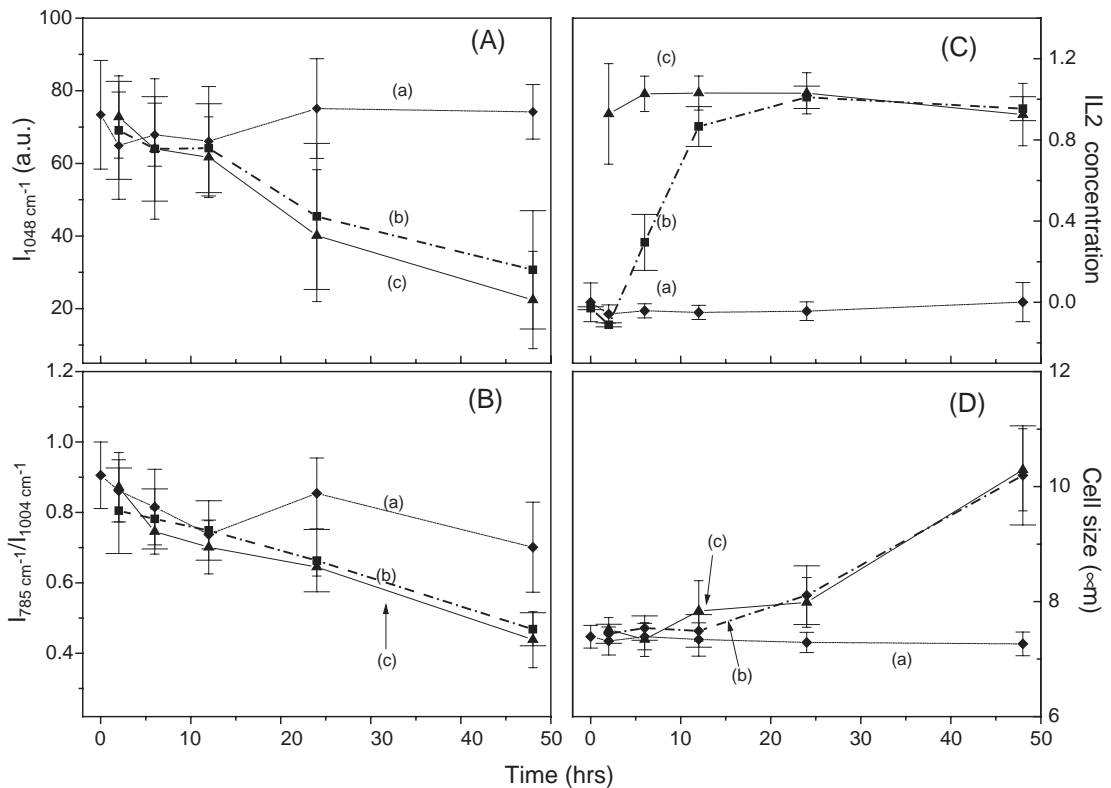


Fig. 2. Activation-dependent alterations in Raman spectroscopic profiles correlated with blastogenesis. RsL.11 T cell were cultured with (a) no stimulus, (b) Con A, or (c) the combination of Con A and IL-2. RsL.11 T cells and supernatants were obtained at the onset of culture (0 h), or were harvested at 2 h, 6 h, 12 h, 24 h, and 48 h. Shown are (A) the averaged intensities of the 1048 cm^{-1} band (I_{1048}), (B) the averaged ratio between the 785 cm^{-1} and 1004 cm^{-1} bands (I_{785}/I_{1004}), (C) IL-2 activity, and (D) average cell diameters as a function of culture duration. Each spectroscopic measure was averaged from 10 separate T cells. One-way ANOVA (Scheffe's post hoc test) revealed significant differences between control (no stimulus) and activated T cells (Con or Con A/IL-2) for measurements of I_{1048} (48 h, $p < 0.001$; 24 h, $p < 0.004$), I_{785}/I_{1004} (48 and 24 h, $p < 0.001$), IL-2 production (48, 24, and 12 h, $p < 0.001$; 6 h, $p < 0.004$), and cell size (48 h, $p < 0.001$; 24 h, $p < 0.003$).

Table 1
Raman bands of RsL.11 T cells and their tentative molecular assignment (Puppels et al., 1990; Peticolas et al., 1996; Notingher et al., 2003)

Band (cm ⁻¹)	Assignment
643	p: C–C twist, Tyr
676	T, G
725	A
785	C, T/DNA:O–P–O ⁻
830	DNA: O–P–O ⁻ /Tyr
852	Tyr
895	DNA bk/deoxyribose
939	p: C–C bk
1004	Phe
1048	C–O str. in deoxyribose
1093	DNA: O–P–O ⁻
1126	C–N str.
1259	Amide III, β sheet
1312	A def
1338	A, G def
1376	T, A, G
1451	Lipids/p: CH def
1581	A, G
1609	Trp/Phe
1660	Amide I, α helix

Abbreviations: A, adenine; G, guanine; C, cytosine; T, thymine; def, deformation; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; str., stretching; bk, backbone; p, proteins.

changes in specific Raman band intensities, cell enlargement per se did not represent the cause of altered band intensities. Con A-activated RsL.11 T cells exhibited some degree of size heterogeneity; thus, we were able to directly compare resting and activated T cells having approximately the same cell diameters (Fig. 3). Size-controlled comparisons of resting (top spectrum) and activated (middle spectrum) T cells nonetheless revealed the characteristic activation-dependent alterations in band intensities at 1048 and 785 cm⁻¹. Activated and rested T cells, however, did not exhibit differences in the magnitude of other control emissions including those at 1004 and 1451 cm⁻¹. These data indicate that the activation-dependent alterations in the Raman spectra were most likely due to the unique intracellular environment of activated T cells rather than due to global aspects of cell size.

The experiments described above focused on the RsL.11 T cell clone. To determine whether these findings were characteristic of T cells in general, thymocytes were isolated and were or were not

activated for 72 h with IL-2 or Con A (Fig. 4). Thymocytes cultured in IL-2 exhibited substantial differences in cell size (Fig. 4B). Large thymocytes were IL-2-responsive and therefore were activated whereas small thymocytes most likely represented the IL-2R⁻ subset that retained a resting phenotype in these cultures. Comparison of these activated (bottom) and resting (top; Fig. 4A) thymocytes revealed activation-dependent decrements in emission at 785, 1093, 1376, and 1581 cm⁻¹. Activated thymocytes also exhibited decrements in band intensities at 676 and 725 cm⁻¹ but did not exhibit alterations in other control emissions (1004, 1451, and 1660 cm⁻¹). Profound differences were also noted in comparisons of non-activated thymocytes (no mitogen, spectrum I of Fig. 4C) and fully activated Con A-stimulated thymocytes (spectrums II and III of Fig. 4C). Activation-dependent decrements in band intensities were noted at several frequencies including those at 676, 725, 785, 1093, and 1376 cm⁻¹. Activation did not affect other emissions at 1004 and 1451 cm⁻¹ but diminished the magnitude of the other control emission at 1660 cm⁻¹. Unlike rested RsL.11 T cells, the emission at 1048 cm⁻¹ in rested thymocytes was negligible and therefore was not a useful activation marker for thymocyte activation. The analysis of fully activated thymocytes was stratified into intermediate-sized cells (curve II) and large blast T cells (Curve III) yet size-dependent differences in the Raman spectra were not evident between these two subsets. These findings indicate that specific Raman frequencies are exquisitely sensitive to the activation status of T cells in general and can be used to gauge the activation phase of individual T cells.

This study focused on CD4⁺ T-helper lymphocytes because this cell type represents a central regulatory subset that controls adaptive immunity. As shown in this study, Raman spectroscopic analysis of individual living T cells reveals a unique spectroscopic profile associated with T cell activation such that the degree of activation can be quantitatively ascertained in isolated living T cells. The molecular basis for activation-decremented band intensities at 676, 785, 1048, 1093, 1376, and 1581 cm⁻¹ is currently unknown. These activation-dependent alterations may reflect altered secondary or tertiary structure of nucleic acids and may possibly reflect a more relaxed

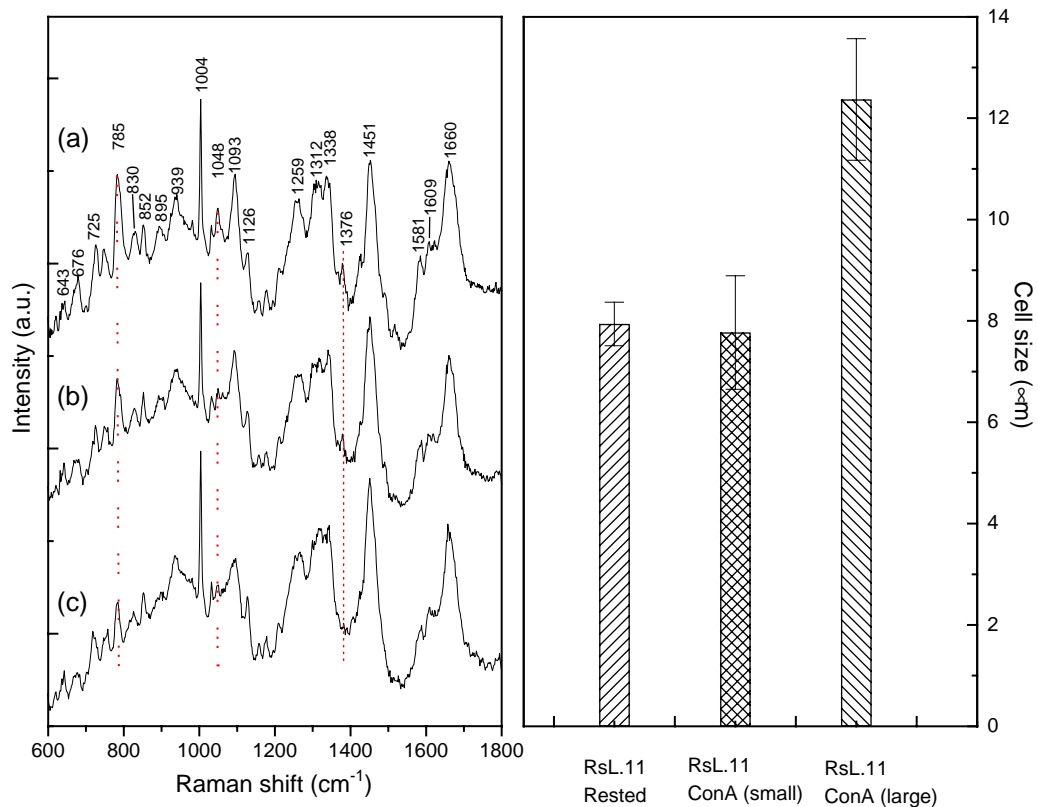


Fig. 3. Activation-dependent alterations in Raman spectroscopic profiles were not strictly a function of cell diameter. Resting RsL.11 T cells were cultured in IL-2 or were activated by a 72-h culture in the presence of Con A and IL-2. Shown are Raman spectra (left) and cell diameters (right) of resting T cells (a) or size-selected small (b) or large (c) activated T cells. Each spectrum was averaged from more than 15 separate T cells.

physical state of DNA associated with active transcription. Alternatively, some band intensities may reflect activation-dependent changes in the oxidation state of key enzymes involved in metabolism of reactive oxygen intermediates.

Raman spectroscopy has been used to study enzymes involved in respiratory burst metabolism, particularly NADPH oxidase system and peroxidases of neutrophilic and eosinophilic granulocytes and other phagocytes (Puppels et al., 1991; Salmaso et al., 1994; Sijtsema et al., 1998, 2000). Upon activation of neutrophils with PMA for example, Raman spectroscopy revealed intracellular reduction of cytochrome *b*₅₅₈ subunit of NADPH oxidase and myeloperoxidase. Intracellular oxidation/reduction status of leukocytes in general appears important for regulation of cellular activation and expression of

effector activity. Like neutrophil activation, T cell activation also influences the intracellular redox potential of T cells, and the redox status of activated T cells in turn controls the balance between survival and apoptosis (Sandstrom et al., 1994).

The study of neutrophil activation by Raman spectral analysis (Otto et al., 1998) showed that the intensity of the 1376 and 1581 cm⁻¹ bands reflected the concentration of oxidized cytochrome *b*₅₅₈ in individual neutrophils. Activation of neutrophils with PMA or *Escherichia coli* or reduction by dithionite treatment resulted in the reduction of oxidized cytochrome *b*₅₅₈ and the disappearance of bands at 1376 and 1581 cm⁻¹ bands coupled with the appearance of bands at 1360 and 1525 cm⁻¹ (reduced cytochrome *b*₅₅₈). Our analysis of RsL.11 T cells also revealed activation-dependent decrements in band

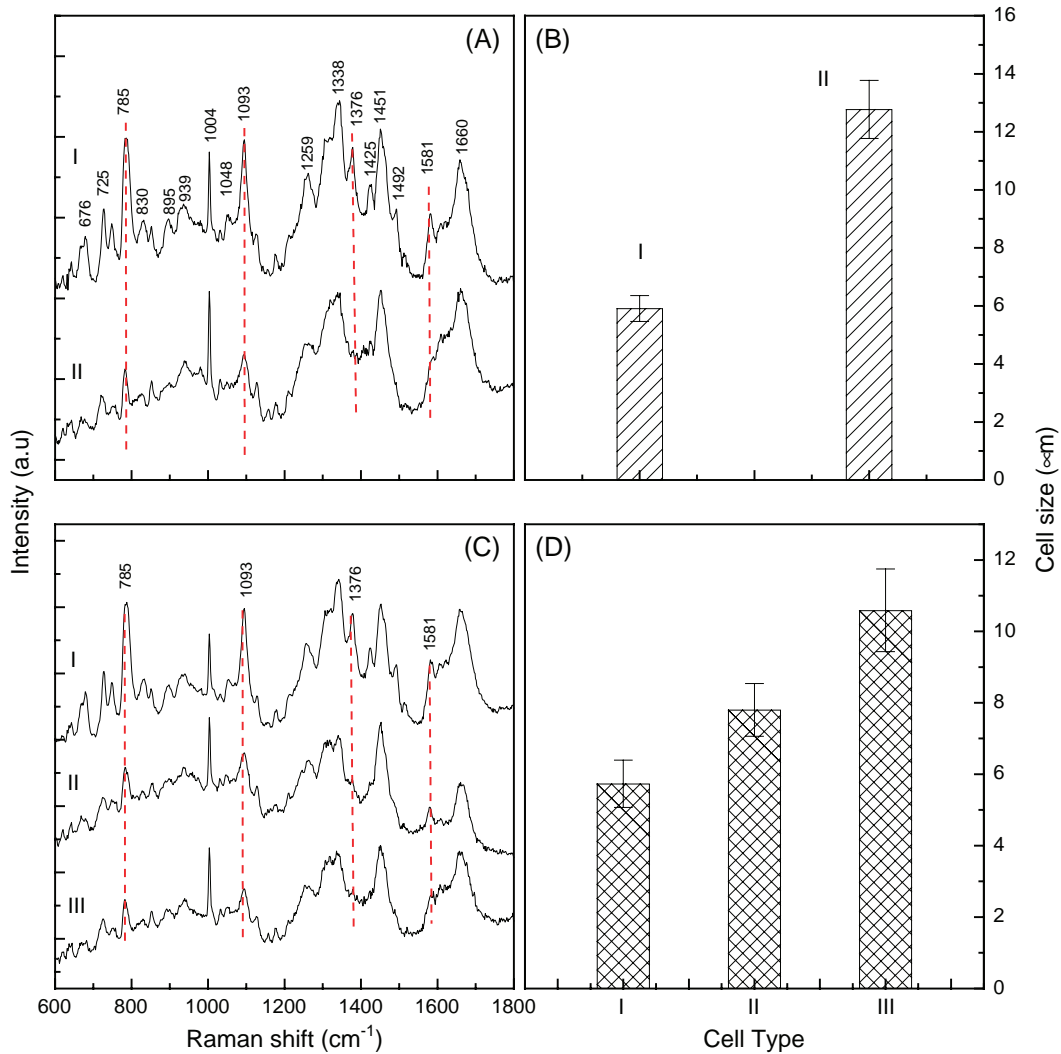


Fig. 4. Unique Raman spectroscopic profiles distinguish rested and activated thymocytes. (A) Thymocytes were cultured for 72 h in the presence of IL-2. Shown are averaged spectra from small IL-2 unresponsive T cells (curve I) or large IL-2 responsive T cells (curve II). (C) Thymocytes were cultured for 72 h in the presence or absence of Con A. Shown are averaged spectra from non-activated thymocytes (curve I) or from Con A-activated T cells (curve II, intermediate-sized T cells or curve III, large T cells). The right panels (B and D) show the corresponding averaged cell diameters. Each spectrum was averaged from more than 15 separate T cells.

intensities at 1376 and 1581 cm^{-1} together with changes in the other bands. These changes may reflect alterations in DNA because the 1376 and 1581 cm^{-1} bands represent well-known DNA-base vibrations (Table 1). Alternatively, T cells, like neutrophils, may also have redox-sensitive enzymes that may be regulated by cellular activation. Activation-dependent decrements in band intensities at 1376 and 1581 cm^{-1} in both T cells and neutrophils may therefore reflect

similar enzymatic activities controlling metabolism of reactive oxygen intermediates.

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