Monitoring human leukocyte antigen class I molecules by micro-Raman spectroscopy at single-cell level

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Abstract. Human leukocyte antigen (HLA) class I molecules are formed by three immunoglobulin-like domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$) once folded by peptide and β_2-microglobulin show the presence of two α-helix streams and one β-sheet limiting the pocket for the antigenic peptide. The loss of HLA class I expression in tumors and virus-infected cells, on one hand, prevents T cell recognition, while on the other hand, it leads to natural killer (NK) cell mediated cytotoxicity. We propose the possibility of using Raman spectroscopy to measure the relative expression of HLA class I molecules at the single-cell level. Raman spectra are recorded for three cell lines (K562, T2, and T3) and monomers (HLA class I folded, unfolded and peptide+β_2-microlobulin refolded) using 830 nm laser line. Our data are consistent with the hypothesis that in the Raman spectra, ranging from 1600 to 1800 cm^{-1}, the intensity variation of cells associated with HLA class I molecules could be measured.

[DOI: 10.1117/1.3368687]

Keywords: cells; Raman spectroscopy; vibration analysis; biomedical optics; statistical optics; laser spectroscopy.

Paper 09548R received Dec. 10, 2009; revised manuscript received Feb. 2, 2010; accepted for publication Feb. 3, 2010; published online Apr. 12, 2010.

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1 Introduction

Human leukocyte antigen (HLA) class I molecules are polymorphic cell-membrane-associated glycoproteins involved in antigen presenting process and recognized by lymphocyte receptors: i.e., T-cell receptor (TCR) in T-cell lymphocytes and killer cell immunoglobulin-like inhibitory receptors (KIR) in natural killer (NK) cells. The HLA class I molecule is a trimeric glycoprotein composed of one heavy chain, the nine amino acid antigen-derived peptide, and one β_2-microglobulin. The heavy chain has three immunoglobulin-like domains and its tertiary structure once folded around the peptide and β_2-microglobulin is characterized by two α-helix and one β-sheet structures that together form the pocket for the immunogenic peptide.

Mismatch in HLA class I antigen haplotypes between a donor and a host is the major cause of transplant rejection.[1] The biological function of HLA class I molecules is to activate T cells. In contrast, the NK cells are inhibited by HLA class I expression on target cell membrane.

The HLA class I molecules are expressed by all cell types. However, in pathological conditions such as virus infection[2,3] or cell transformation, the membrane levels are reduced.[4–6]

The decrease in HLA class I, on one hand, prevents T-cell recognition of virus-infected or -transformed cells, while on the other hand, it triggers NK-mediated cytotoxicity.[7] Therefore, it is of great interest to monitor the MHC class I expression in patients with neoplastic disease or virus infection to set the most appropriate therapeutic intervention.

There are many spectroscopic techniques [fluorescence, nuclear magnetic resonance (NMR), x-rays diffraction, etc.] that are currently being used for the analysis of biological cells to understand their chemical structure and conformational changes in different pathological conditions.[8,9] Vibrational spectroscopy techniques and, in particular, Raman spectroscopy are proved to be effective in analyzing cells or biological compounds.[10,11] The advantage of this technique over other traditional spectroscopic techniques is given by a very low scattering cross section for water, thus, enabling the understanding of the chemical structure of the specimen to the level of vibrational transitions. Moreover, it is a nondestructive technique that provides information at the molecular level related to DNA, proteins, lipids, etc. within the cell in its spectral range of 700 to 1800 cm^{-1}.

Since the relative quantitative and qualitative information on HLA class I gene products are relevant in different clinical settings and given the possibility of utilizing Raman spectroscopy to understand the secondary structure of glycoprotein, we set out to monitor the HLA class I expression on the cell membrane at the single-cell level.

Using HLA class I specific experimental systems we produced evidence supporting our research hypothesis, demonstrating that the relative Raman intensity variation in the spectral region spanning from 1600 to 1800 cm^{-1} for different cells could be associated to HLA class I molecules. The analysis of Raman spectra using principal component analysis (PCA) enabled us to differentiate various cells with different HLA molecular concentration. PCA is a multivariate method of analysis whose main concern is to reduce the dimensionality of a multidimensional data set comprising a large number of variables, while retaining those characteristics of the original data set that contribute most to its variance. It let us classify or categorize Raman spectra data that otherwise would be impossible to distinguish simply with visual examination. Different subsets were observed on the basis of a dissimilarity function (Euclidean distance function) using cluster analysis.

2 Experiments

2.1 *Materials and Methods*

2.1.1 *Cell lines*

Three cell lines were taken into consideration for Raman analysis, namely, K562 (K562, human erythroleukemia); T2 (T2 cell line is a human cell hybrid with a defect in antigen peptide transporter (TAP) gene expression[12]); and its related transfectant T3 [obtained by human TAP1+2 cDNA (complementary DNA) transfection into the T2 cell line]. All cell lines were cultured at 37 °C in 5% CO_2 atmosphere in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 5% heat-inactived fetal bovin serum (FBS) (Sigma-Aldrich, Milan, Italy) and 2 mM L-glutamine (Invitrogen, Milan, Italy). The T3 cultures were supplemented with G418 at a concentration of 1 mg/ml (Invitrogen, Milan, Italy).

Interferon-gamma (IFN-γ) treatment. IFN-γ is a dimerized soluble cytokine, the only member of the type II class of interferons. It is a cytokine critical for innate and adaptive immunity against viral and intracellular bacterial infections and in tumor control. IFN-γ is secreted by Th1 cells, Tc cells, dendritic cells, and NK cells. IFN-γ receptors trigger STAT1 transcriptional activity in treated cells that leads to major histocompatibility complex (MHC) class I heavy chain translation and traduction that results in increased levels of the membrane-associated molecules.

The K562 cells were incubated with IFN-γ for 48 and 72 h. After the cytokine treatment, MHC class I expression levels on the K562 cell surface were measured by indirect immune-fluorescence staining and FACS (fluorescence-activated cell sorter) analysis using the FACS Calibur System (BD Biosciences).

T2 peptide pulsing. T2 cells were incubated with 200 μM of peptides overnight at 37 °C in a humidified 5% CO_2 atmosphere. The absence of TAP genes leads to the cell expression of empty MHC class I molecules that are highly unstable. By exogenous antigen peptide pulsing it is possible to stabilize and increase the MHC class I cell membrane levels.

The sequence of peptide A2 is NH_2-FLPSDFFPSV-*COOH* with a molecular weight of 1155.32 (Primm, Milan, Italy).

2.1.2 *MHC class I heavy chain and β_2-microglobulin chain*

Single colonies from glycerol stocks of the bacterial strains containing the protein (pET-3d encoding exon 2, 3, 4 of the HLA A*0201 molecule or exon 2, 3, 4 of the HLA B*0701 molecule, both in-house constructs, and pET-3d encoding the β^2m human molecule, in-house construct) were grown on Luria-Bertani medium plates (Sigma) supplemented with 100 mg/ml of ampicillin, then inoculated in the same medium. The cultures were incubated at 37 °C with constant mixing until they reached an optical density (OD) of 0.4 to

0.6 at 550 nm. The expression of the protein was then induced by adding 10 ml of 200 mM IPTG (Alexis Biochemicals), and the culture was grown further for 4 h to allow maximum protein expression. At the end of 4 h, the bacteria were collected via centrifugation and the resulting pellet was resuspended in ice-cold phosphate-buffered saline (PBS) and frozen at −70 °C overnight.

Purification of inclusion bodies. The pellet was defrosted and sonicated eight times at maximum amplitude. The resulting solution was washed three times with freshly made Triton wash buffer [50 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.1% sodium azide, 0.5% Triton X100, 1 mM dithiotreitol (DTT)]. A further wash step was performed using freshly made resuspension buffer (50 mM Tris HCl pH 8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT). The resulting pellet was dissolved in a minimum necessary volume of an 8M urea-based buffer and left to dissolve at 4 °C rolling overnight. After overnight incubation, the insoluble material was removed via centrifugation and the concentration of the protein in the solution was assessed using the Micro BCA kit (Pierce).

SDS-Page gel. We collected 10 μl of the samples prior to and during the induction and loaded them on the gel together with an equal volume of loading buffer. The samples and loading buffer were denatured for 3 min at 98 °C, then loaded, alongside, with a molecular weight standard (Biorad Laboratories). The gel was run for 1 h at 150 V and 400 mA in 25 mM Tris HCl, 250 mM glycine, 0.1% SDS. At the end of the run, the gel was stained in Coomassie blue for 15 min, destained overnight in methanol and acetic acid buffer, and dried.

MHC-peptide refolding. The refolding of MHC-peptide complexes was performed using a rapid dilution method, as follows. While working at 4 °C, 4.8 mg of β_2m, 6.2 mg of A2 chain, and 2 mg of peptide (AVIDNFVKKL for A*0201, TPRTGGGAM for B*0701) were added drop by drop to 200 ml of refolding buffer [100 mM Tris HCl pH 8, 400 mM arginine monohydrochloride (Sigma), 1 mM EDTA pH 8, 5 mM reduced glutathione, 0.5 mM oxidized glutathione]. The solution was left for stirring at 4 °C for 48 h and then concentrated to 2 ml using the UltraFiltration Cell Amicon (Millipore) system and then Vivaspin tubes. The concentrated solution was stored at 4 °C before performing fast protein liquid chromatography (FPLC) and eluted with low-salt buffer (10 mM Tris-HCl, 5 mM NaCl, filtered and degassed). Fractions containing the correctly refolded peptide-MHC complexes were concentrated to 1 ml, then either stored at 4 °C for short-term use or aliquots were made and stored at −70 °C for long-term use.

HLA class I molecules. A*0201 was obtained as described by Mathieu et al., 2007,[13] while the B*0201 refolding was obtained by a standard cytomegalovirus (CMV) epitope obtained from CMV protein pp65, well known to bind to HLA-B molecules. The HLA class I refolded molecular weight (A*0201 or B*0701+β^2m+peptide) is approximately 40 KDa.

2.2 *Characterization Techniques*

2.2.1 *Flow cytometry and monoclonal antibodies*

In the indirect staining experiments, as a first step, cells were preincubated with human serum for 15 min, and isotype-matched controls were used to set up the negative values. Then the cells were incubated with the appropriate monoclonal antibody (mAb), followed by second-step incubation with FITC-conjugated goat anti-mouse secondary IgG (immunoglobulin G) antibody (Jackson Immuno Research Baltimore, Maryland). For the indirect immunofluorescence assays, the specific anti-human HLA class I mouse mAb W6/32 (BD PharMingen, Mountain View, California) was used.

Samples were analyzed by the (FACS FACSCalibur, model: BD Biosciences) and the acquired cytofluorographic data were analyzed using Cellquest software (BD Biosciences).

2.2.2 *Micro-Raman spectroscopy*

K562, K562 treated with IFN-γ tested in time course experiments, T2, T3, and T2 pulsed with HLA-A2 specific peptides and monomers (HLA-class I folded and unfolded heavy chains) were used for Raman measurements. Four mapping measurements were carried out for each cell type (cell preparation for each cell type was made at least three times and thereafter the respective analytical measurements were performed) to ensure the reproducibility of the results. We deposited 2 μl of cell suspension on gold-sputtered based substrate, kept in a closed microcryostat chamber. The sample was left in the chamber at room temperature for 5 to 10 min to eliminate the excess water.[14] Note, here, that the samples were freshly analyzed when the cells were in the hydrated state and not in the dried state.[14]

Microprobe Raman spectra were excited by a near-IR laser with a 830 nm laser line in backscattering geometry through a 50× long range objective (NA=0.50) in the range of 700 to 1800 cm^{-1}. The laser power was held constant at 65 mW for all the cells, except for monomers where a 13-mW laser power was used instead. The substance was always checked through optical image before and after the measurements. The gold-sputterd substrate with drop coating deposition (DCD) protein samples were kept in a closed microcryostat chamber (Linkam Hot/cold cell) to avoid impurity from the surroundings. The micro-Raman spectrometer was calibrated before the cell measurement by using a Si wafer as a reference. All the Raman spectra were, first, baseline-corrected using four to five-order polynomials to eliminate the autofluorescence background and were, then, normalized to a protein marker band centered at 1450 cm^{-1}, attributed to C—H_x bending vibration.[15,16]

Raman line-mapping measurements were performed using inVia Renishaw Microscope by selecting around 15 ×15 μm^2 on each cell pellet deposited on the substrate. About 10 to 18 Raman spectra from the central part of the cell's mapping area were chosen for analysis. The spectra were then smoothed using the nine-points Savitzky-Golay method and averaged for Raman analysis. The secondary structure [α-, β-, and random coils (γ)] contributions were estimated on the deconvolution of the spectra in the range between 1620 and 1700 cm^{-1}. The secondary structure was

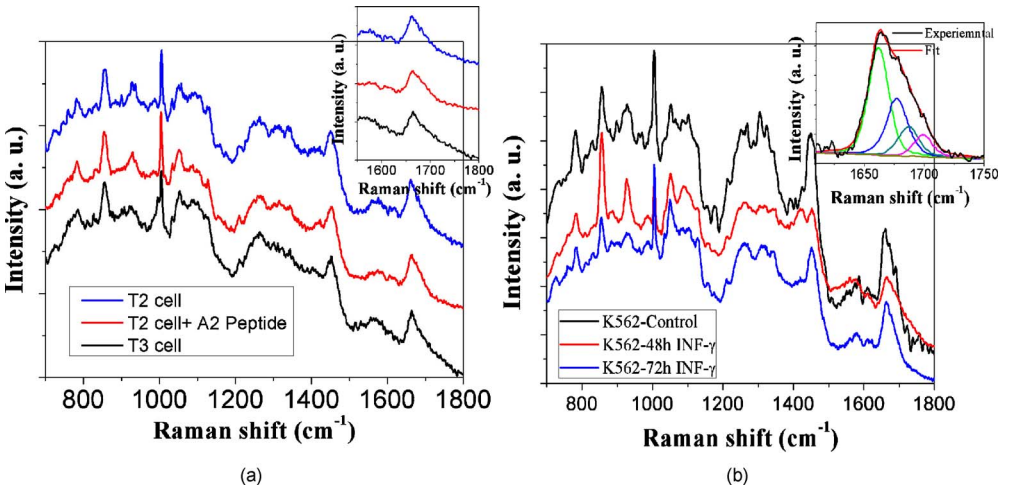

Fig. 1 Micro-Raman spectra of (a) T2, T3, and T2 cells treated with A2 peptide in the range of 700 to 1800 cm^{-1}, and in the inset, the zoomed area of the secondary structure; and (b) the K562 cell line induced with IFN-γ by varying time periods, and in the inset, the deconvolution of the spectrum using mixed distribution function [$MG+(1-M)L$, M, weight factor].

evaluated by carrying out deconvolution for one-by-one cell Raman spectrum and thereafter standard deviation was calculated. The deconvolution of spectra was performed by fixing the bandwidth and peak position, which differentiates our deconvolution procedure from simple spectral fitting.

PCA was performed for all the cell lines and was separated into two graphs for sake of clarity: (1) for K562 cells and derived cells and (2) T series cell lines, using MATLAB. PCA analysis involves the calculation of principal components (PCs) that describes the greatest variance of the spectral data from the mean; 41 PCs were calculated for the purpose of this study. Each of the protein spectra in the data set can be reconstructed from the 41 PCs, by multiplying each PC by a different variable, known as score. As the PCs are common for all the spectra in the data set they can be put aside, enabling each spectrum to be represented by its 41 scores. This enables a massive reduction in the data points that need to be processed, with a minimal reduction in functional information. Cluster analysis was finally performed to show the clustering in different groups. The cluster analysis showed about 13% error in grouping the experimental data.

3 Results and Discussion

Different amounts of MHC class I molecules in three different experimental configurations [K562 cell line treated with MHC class I inducing cytokine[17,18] (IFN-γ); the T2 cell lines defective for antigen peptides transporters (TAP); and the related T3 cell lines, obtained by TAP gene transfection in T2] were used to assess the possibility to differentiate the HLA class I membrane-associated levels at a single cell level, using Raman spectroscopy.

The restoration of peptide transporter activity leads to a highly stable trimolecular complex on T3 cell surface. Thus, T2 and T3 cells express low and high MHC class I molecules associated membrane, respectively.[19] The trimeric MHC class I complex could be restored on T cells by antigen-derived peptide cell pulsing. We used HLA-A2-specific peptide[20] since T2 expresses HLA class I molecules as unstable dimers on its cell surface. The specific peptide loading stabilizes the HLA class I molecules on the T2 cell membrane, increasing their expression levels. All these systems were, previously, used to modulate positively and selectively the MHC class I expression on a cell membrane. In our study, the induction of MHC class I expression in all these experimental systems was monitored by immunofluorescence and the FACS technique and compared with Raman spectroscopy analysis.

Micro-Raman measurements were carried out on the K562 cell line and on K562 cell line treated with IFN-γ at different times in PBS. Figures 1(a) and 1(b) show average cell Raman spectra in stacked order, namely, K562 control, K562 under IFN-γ treatment for 48 h and 72 h, T2, T2+HLA-A2 peptide, and T3 cell, in the range between 700 and 1800 cm^{-1}. This range consists of information about various amino acids, DNAs, lipids, and, in particular, the secondary structure of the cell proteins.

3.1 *HLA Class I Induction by IFN-γ on the K562 Membrane Surface*

Raman spectra obtained from K562 and other cells (time course treatment using IFN-γ) are similar to the spectra of most living cells.[21] All the spectra show different Raman peaks at about 780, 850, 1004, 1125, 1450, and 1660 cm^{-1}. Typical bands in these spectra [see Fig. 1(a)] are originated from nucleotide conformation (700 to 800 cm^{-1}), skeleton geometry and phosphate-related vibrations (800 to 1200 cm^{-1}), nucleotides (1200 to 1600 cm^{-1}), and C—C and C—H_x modes due to proteins and lipids (about 1450 cm^{-1}). Also, amide vibrations, such as amide-I band (due to C═O stretching, 1650 to 1700 cm^{-1}) and amide III band (due to C—N stretching, and N—H bending, about 1250 cm^{-1}) in proteins are easily identifiable. The amide I bands in the range between 1620 and 1700 cm^{-1} also provide very important information about the conformation of secondary structure [α-helix, β-sheet, and random coil (γ) structure] of proteins. Different amino acids are recognized explicitly at about 1004, 1011, and 1032 cm^{-1} in the Raman spectra. The cell's conformational changes due to IFN-γ treatment can be observed by intensity variation for the bands centered about

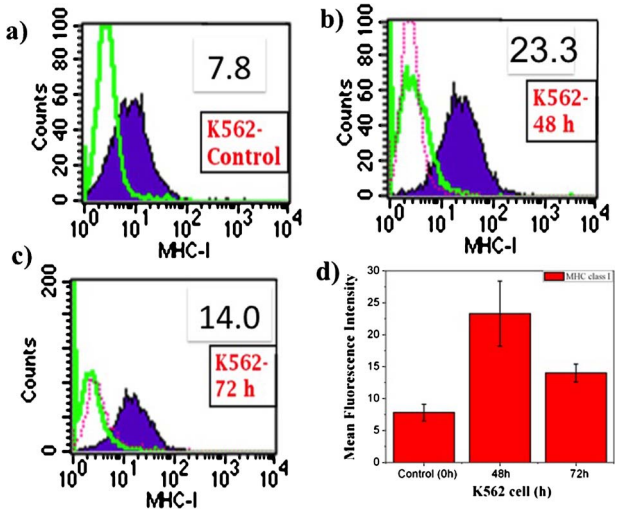

Fig. 2 FACS results of three K562 cell series: (a) K562 control, (b) K562+IFN-γ for 48 h, and (c) K562+IFN-γ for 72 h, and (d) mean fluorescence intensity (MFI) of HLA class I expression on these cells [standard deviation (SD) is shown as an error bar].

725, 855, and 1004 cm^{-1} (DNA bases and amino acid), whereas profile variation can be observed for the bands centered at 1450 cm^{-1} [C—H_x deformation vibration (associated to DNA, protein, and lipid)], and in the range of 1625 to 1750 cm^{-1} (related to the proteins' secondary structure). In the inset of Fig. 1(a), the zoomed area of Raman spectrum in the range of 1620 to 1740 cm^{-1} is shown. To avoid interference between the peaks during deconvolution, all the spectra were fitted after fixing the peak position and spectral linewidth. The three fitting curves, observed at around 1660, 1677, and 1686 cm^{-1}, are related to α, γ, and β secondary structure.

As previously described,[21–23] 48 h IFN-γ treatment of K562 induced a clear up-regulation of MHC class I molecules detected by the immunofluorescence technique, while at 72 h, the MHC class I levels start returning to the baseline levels. Similar findings were observed by FACS measurements, shown in Figs. 2(a)–2(d). Figure 2 clearly shows the up-regulation of HLA class I levels after treatment with IFN-γ for 48 h, and after 72 h there was down-regulation of HLA class I on the cell surface. The mean fluorescence intensity MFI of HLA class I by immunofluorescence technique is 7.8, 23.3, 14.0 for control, 48 and 72 h, respectively. The MFI values for different cells with the standard deviation as an error bar are shown in Fig. 2(d). The effect of IFN-γ on the K562 cell line with time leads to the variation in HLA class I levels, as observed by FACS, and in parallel there was an alteration in secondary structure observed by micro-Raman spectroscopy, which is compared and shown in Fig. 2(d) and 3(a). The Raman spectrum of the HLA class I monomer shows a dominant band attributed to α-helix at about 1655 cm^{-1}. The treatment of cells with IFN-γ causes first a concomitant up-regulation of HLA class I on the cell membrane and Raman α-helix content (and consequently the decrease in β-sheet/α-helix and/or random coil/α-helix ratio with respect to the K562 control cell: considering the β-sheet and random coil not variable in percentage of total secondary structure), respectively. On the other hand, after 72 h, with IFN-γ treatment of K562, we observed a reduction in HLA class I expression by immunofluorescence technique and an increase of the β-sheet/α-helix and/or random coil/α-helix ratio with respect to the K562 cell after 48 h IFN-γ treatments. Figure 3(a) shows the ratio of β-sheet/α-helix (β/α) and random coil/α-helix (γ/α) by varying the time (h). However, in both cases, the ratio decreased for K562 cells treated for 48 h with IFN-γ and, thereafter, an increase in the ratio values for K562 cells treated with IFN-γ for 72 h. The decrease in ratio confirms an increase in α-helix structure on cell membrane, observed by FACS findings because the presence of IFN-γ induces the increase of MHC I peptide with time. PCA was performed in the range between 700 and 1800 cm^{-1} to categorize and then to distinguish the cells on statistical bases. It is a statistical method whereby the information content of each spectrum is described by a limited number of variables (the PCs). A PCA scatter plot (PC2 versus PC3) is shown in Fig. 3(b). Raman spectra, as observed in Fig. 1, did not clearly show much difference from each other. The PCA analysis [shown in Fig. 3(b)] makes them clearly distinguishable from each other. Moreover, the 3-D PC (PC1 versus PC2 versus PC3) plot is also shown in Fig. 3(c). The 3-D-PCA-plot curve shows an explicit distinction of these different cell types. The loadings curve corresponding to PC1, PC2, and PC3 are also presented in Fig. 4 to further substantiate the analysis. The first principal (PC1) loading curve with spectral variance of 79.5% mostly shows dissimilarities in the region of secondary structure (1600 to 1700 cm^{-1}), at around 1050 cm^{-1}, around 840 cm^{-1}, etc. The PC2 loading curve, with spectral variance of 10.8%, shows remarkable variance in the range of 1150 to 1300 cm^{-1}, and peaks at around 1460 cm^{-1}, 760 cm^{-1}, etc., whereas the PC3 loading curve with a spectral variance of less than 2% shows the major dissimilarities at around 1230 cm^{-1}.

3.2 *HLA Class I Induction for T2 and T3 and the Derived Cell Line*

Since IFN-γ has pleiotropic effect on genes other than MHC class I, we set out to monitor HLA class I molecules using Raman spectroscopy analysis in a more controlled and specific system; the T2/T3 experimental cell system, and in exogenous peptide pulsing experiments using the T2 cell line as peptide presenting cells.

FACS measurements were carried out to assess the relative HLA class I expression in various conditions with respect to the T2 cell line. The results show that HLA class I is highly expressed in the T3 cells, while the T2 cell line has a low residual amount of the molecules on their cell membrane. However, the T2 cells pulsed with exogenous HLA-A2-specific binding peptide, potentially increased the HLA class I expression on T2 cells [Fig. 5(a)]. Taking together, the results show a greater amount of HLA class I expression in T3 cells (TAP genes transfected cells) and in the T2 cell line pulsed with HLA-A2 specific peptide than the T2 cell line, as shown in Fig. 5(a). The MFI of HLA class I expression was about 190 for T3 cells pulsed with HLA-A2 specific peptides with respect to the control HLA class I values detected on T2 cells treated with the complete medium (MFI being 62).

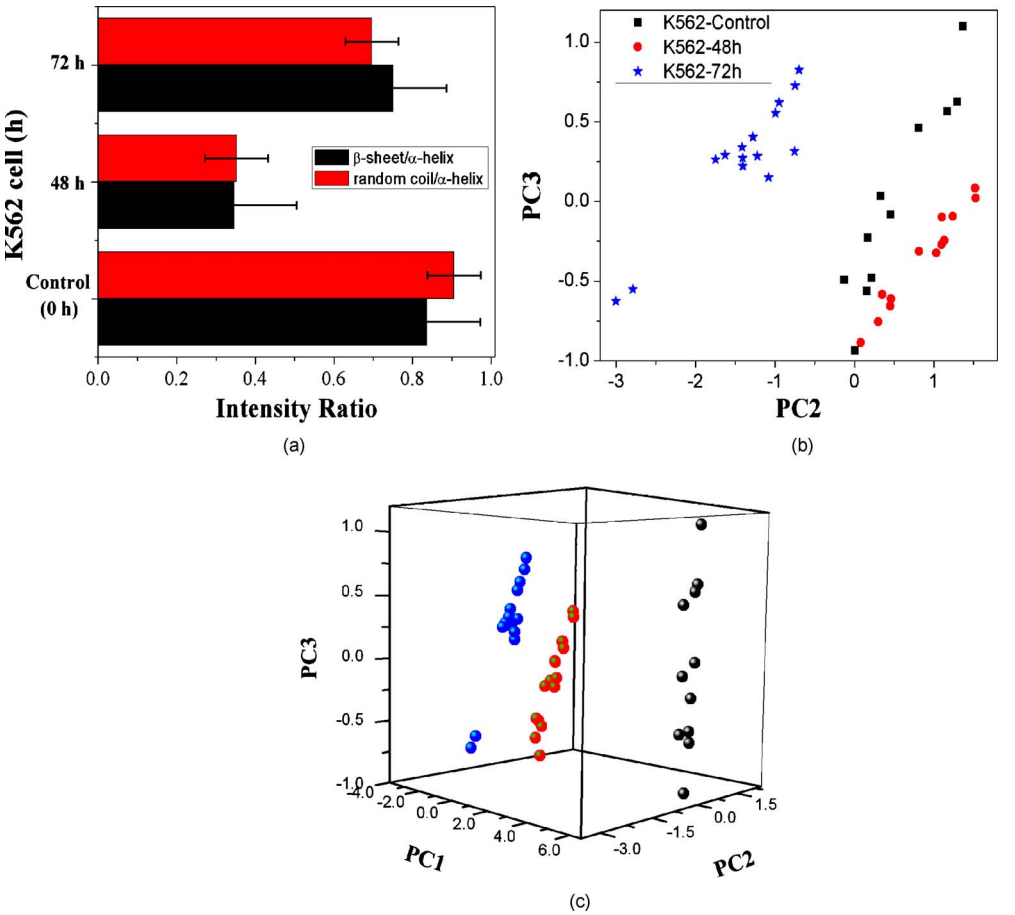

Fig. 3 (a) Ratio of β-sheet/α-helix and random coil/α-sheet versus K562 cell treated with IFN-γ by varying time (h) (SD is shown as an error bar), (b) principal component analysis (PC2 versus PC3) for these three K562 cells, and (c) 3-D PCA plot (PC1 versus PC2 versus PC3) is for the K562 cell line.

Raman spectral analysis confirms the FACS data of HLA class I MFI trend regarding variation in HLA class I, as observed in Fig. 5(b). Figure 5(b) shows that the ratio of random coil (γ) to α-helix (α) secondary structure, first, decreases for T3 cells with respect to T2 cells and, thereafter, an increase was observed with the induction of peptide A2, which induces the HLA class I. Since HLA class I carries α-helix content, the increase in HLA class I causes a decrease in the ratio between random coil and α-helix. Further analysis was carried out to strengthen our findings. Figure 6(a), presents a PCA scatter plot for PC1 against PC3. PCA analysis again distinguishes cell types from one another [Fig. 6(a)], which were similar in Raman spectra (Fig. 1). The results show that the induced HLA class I in the cell line can be clearly differentiated by combining Raman spectra and PCA multivariate analysis. Further, 3-D PCA plot is also shown in Fig. 6(b). Cluster analysis was performed using the Euclidean distance function on these PCs, as shown in Fig. 6(c). On the basis of the number of points belonging to their own group, we clusterted data with an error of around 13%. Figure 7 shows loading curves (PC1, PC2, and PC3) with a maximum variance around 63% (PC1). The figure shows that the maximum dissimilarity is observed in secondary structure region, the region around 1100 cm^{-1}. These results show that Raman spectroscopy results follow the trend of what has been observed by FACS, which is a well-known biomedical cell-screening technique.

Furthermore, Raman measurements were performed on HLA-A2 monomer, HLA unrefolded, and HAGE refolded in the range between 700 and 1800 cm^{-1}, as shown in Fig. 8. The inset zoomed area shows the secondary structure in the range between 1600 and 1800 cm^{-1}. The Raman spectra in

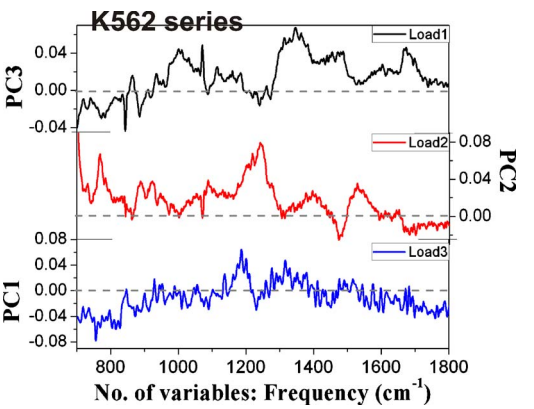

Fig. 4 Loading curve for three PCs (PC1, PC2, and PC3) for the K562 cell lines.

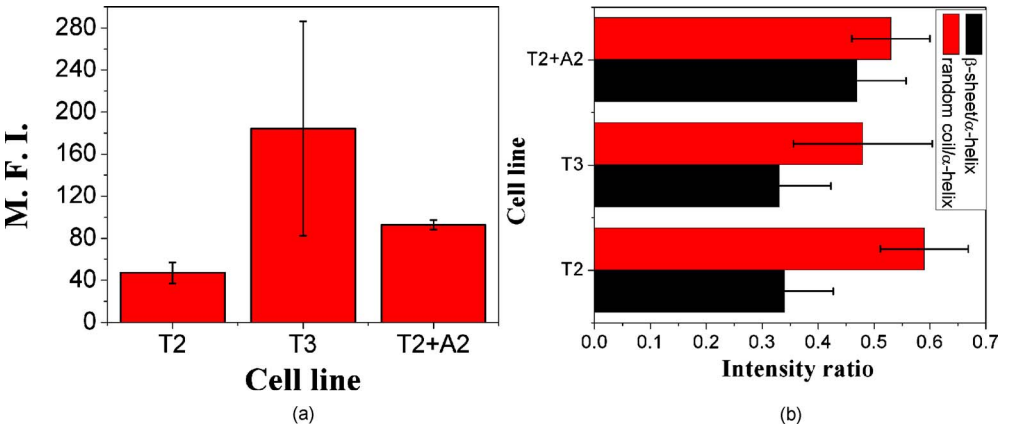

Fig. 5 (a) Mean fluorescence intensity variation in T2, T3, and T2 cells treated with A2 peptide and (b) the ratio of β-sheet/α-helix and random coil/α-helix versus T2, T3, and T2 cells treated with A2 peptide, derived from Raman spectra.

Fig. 8 shows clear presence of phenylalanine (Phe) centered at 1005, 1033, and 1550 cm^{-1}, the P—O stretching vibration due to Tris buffer at about 1080 cm^{-1}, and the COO^- bending centered at about 767 cm^{-1}. The broad band in the region of 1600 to 1720 cm^{-1} is associated to the secondary structure of the monomer. We show clear evidence that the sharp peak reduced drastically for HLA-A2 unrefolded peptide with respect to the HLA-A monomer and again it begins to recover with HAGE refolding. The behavior of this band height variation is due to the change in α-helix structure of monomer.

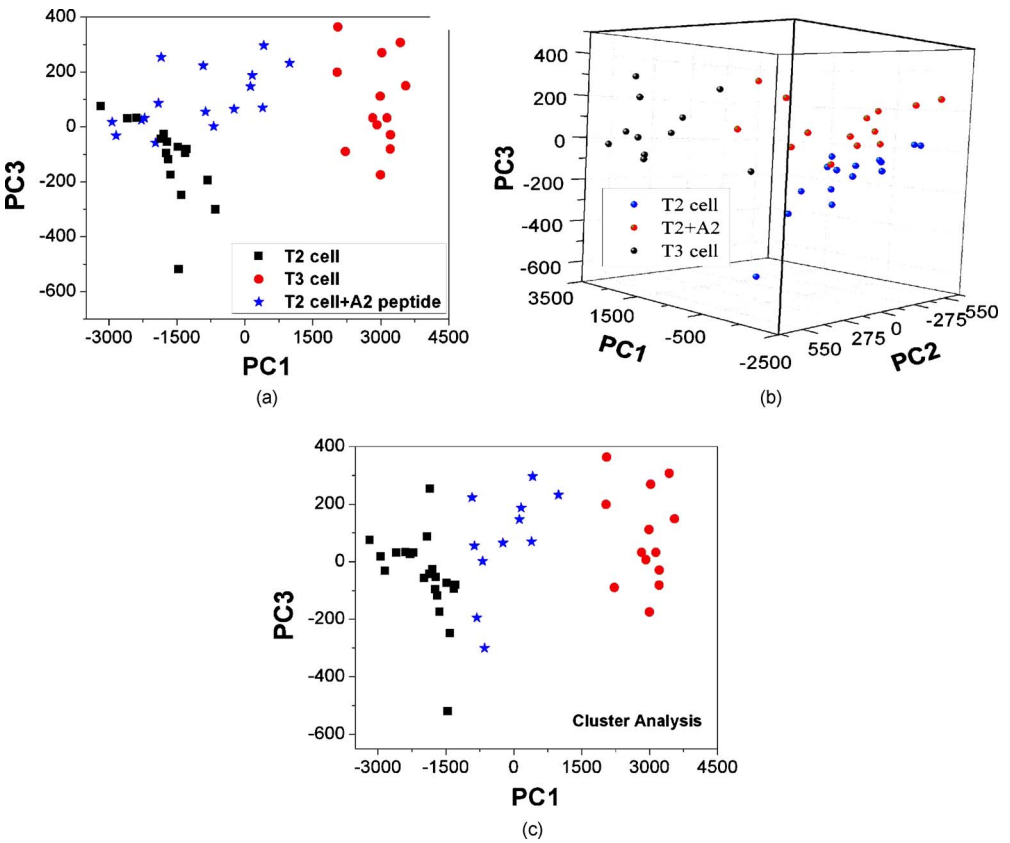

Fig. 6 (a) PCA (PC1 versus PC3), (b) 3-D PCA plot (PC1 versus PC2 versus PC3), and (c) cluster analysis on the micro-Raman data extracted for T2, T3, and T2 treated with the A2 peptide.

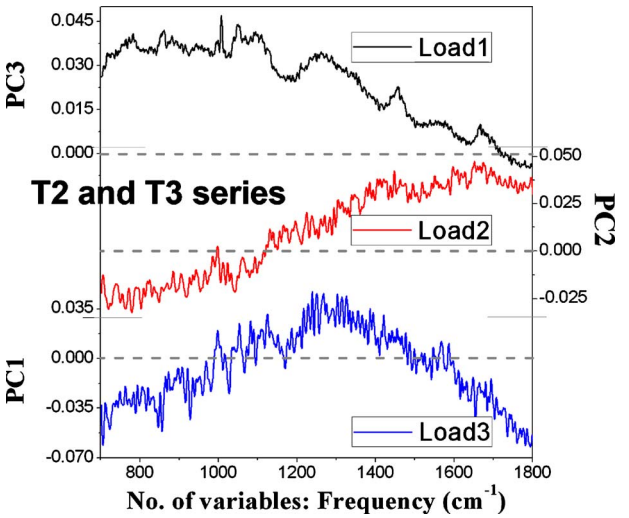

Fig. 7 Loading plot for three primary PCs (PC1, PC2, and PC3) for the T2 cell line series.

Therefore, we can conclude that the Raman spectroscopy follows the conformational changes of monomers, based on its secondary structure.

4 Conclusions

We carried out near-IR (NIR) micro-Raman spectroscopy measurements for various cell lines (K562, T2, and T3 and under several MHC class I modulating treatments). Here, we monitored the MHC class I induction on K562 cell membrane after IFN-γ treatment for 48 and 72 h. The decrease in secondary structure (γ/α and β/α) with respect to the K562 control cell confirms an increase in MHC class I. Furthermore, the K562 data were challenged in more stringent conditions: monitoring HLA class I expression utilizing T2, T3, and T2+A2 binding peptides. In summary, our data clearly showed that in all the experimental settings used, Raman spectroscopy analysis of the cell membrane was able to detect the relative changes of HLA class I expression levels. Raman spectroscopy was used for the first time at the best of our knowledge to monitor relative quantitative HLA class I expression, which is an important molecule in regulating the immune response. We believe that Raman spectroscopy could be a new and useful approach in HLA class I measurements in several contexts:

1. Raman spectroscopy can be used to monitor the HLA class I expression defect in pathological conditions, i.e., infected or transformed tissue-derived cell suspensions. The very limited number of cells required for Raman spectroscopy compared to the numbers required for FACS analysis could result in a more convenient analytical approach in diseases where the biological samples are not abundant.
2. Raman spectroscopy could be used at the molecular level to screen the binding affinity of antigenic peptides library specific for a given HLA class I molecule.
3. Note that the data presented here can have a high impact in terms of monitoring the immune response in infectious diseases or in neoplastic patients by measuring the binding of synthetic HLA class I tetramers presenting immunogenic peptides derived from virus- or tumor-associated antigens to patient-derived T cell clones. This approach will help us to follow the patient immune response during the natural history of disease and/or the response to the vaccination.[25]
4. Raman spectroscopy can also be used to analyze the amino acid changes due to the HLA class I genetic polymorphism and therefore can be used for HLA class I typing of donor organs during transplantation.

In summary, Raman spectroscopy was used, for the first time to the best of our knowledge, to monitor and evaluate the relative variation in HLA class I, which is an important molecule in regulating the immune response.

Raman microspectroscopy was proved to be an effective and promising technique for monitoring cell conditions with good accuracy. In combination with a very well assessed technique such as FACS, it may provide unattainable information about very small changes occurring on cell surfaces.

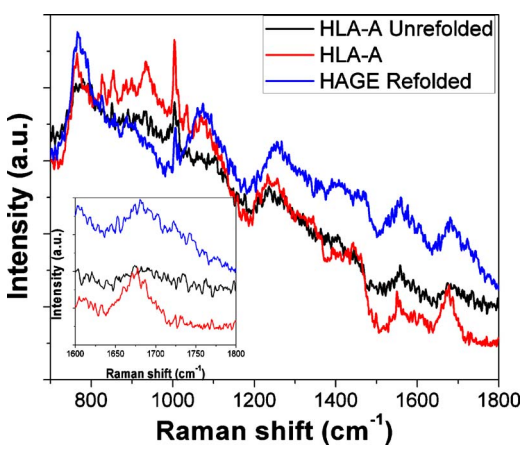

Fig. 8 Micro-Raman spectra for HLA-A monomer unrefolded, HLA-A monomer, and HAGE refolded in the range of 700 to 1800 cm^{-1}. In the Inset, the zoomed area of the plot in the region of 1600 to 1800 cm^{-1}.

References

1. A. Velardi, L. Ruggeri, A. Mancusi, E. Burchielli, K. Perruccio, F. Aversa, and M. F. Martelli, "Immunobiology Working Party of the European Group for Blood and Marrow Transplantation (EBMT). Clinical impact of natural killer cell reconstitution after allogeneic hematopoietic transplantation," *Semin. Immunopathol.* **30**(4), 489–503 (2008).
2. V. M. Braud, P. Tomasec, and G. W. Wilkinson, "Viral evasion of natural killer cells during human cytomegalovirus infection," *Curr. Top. Microbiol. Immunol.* **269**, 117–129 (2002).
3. R. J. Boyton and D. M. Altmann, "Natural killer cells, killer immunoglobulin-like receptors and human leucocyte antigen class I in disease," *Clin. Exp. Immunol.* **149**(1), 1–8 (2007).
4. S. Ferrone and F. M. Marincola, "Loss of HLA class I antigens by melanoma cells: molecular mechanism, functional significance and clinical relevance," *Immunol. Today* **16**(10), 487–494 (1995).
5. T. Kageshita, S. Hirai, T. Ono, D. J. Hicklin, and S. Ferrone, "Down-regulation of HLA class I antigen-processing molecules in malignant melanoma," *Am. J. Pathol.* **154**(3), 745–754 (1999).
6. D. J. Hicklin, F. M. Marincola, and S. Ferrone, "HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story," *Mol. Med. Today* **5**(4), 178–186 (1999).
7. L. L. Lanier, "NK cell recognition," *Annu. Rev. Immunol.* **23**, 225–274 (2005).
8. P. Selenko, Z. Serber, B. Gadea, J. Ruderman, and G. Wagner, "Quantitative NMR analysis of the protein G B1 domain in Xenopus laevis egg extracts and intact oocytes," *Proc. Natl. Acad. Sci. U.S.A.*

103(32), 11904–11909 (2006).
9. K. M. Kemner, S. D. Kelly, B. Lai, J. Maser, E. J. O'Ioughlin, D. Sholto-Douglas, Z. Cai, M. A. Schneegurt, C. F. Kulpa, Jr., and K. H. Nealson, "Elemental and redox analysis of single bacterial cells by x-ray microbeam analysis," *Science* **306**(5696), 686–687 (2004).
10. P. Crow, J. S. Uff, J. A. Farmer, M. P. Wright, and N. Stone, "The use of Raman spectroscopy to identify and characterize transitional cell carcinoma in vitro," *BJU Int.* **96**(9), 1232–1236 (2004).
11. J. W. Chan, D. S. Taylor, T. Zwerdling, S. M. Lane, K. Ihara, and T. Huser, "Micro-Raman spectroscopy detects individual neoplastic and normal hematopoietic cells," *Biophys. J.* **90**(2), 648–656 (2006).
12. G. Terrazzano, M. F. Romano, M. C. Turco, S. Salzano, A. Ottaiano, S. Venuta, S. Fontana, C. Manzo, S. Zappacosta, and E. Carbone, "HLA class I antigen downregulation by interleukin (IL)-10 is predominantly governed by NK-kappaB in the short term and by TAP1+2 in the long term," *Tissue Antigens* **55**(4), 326–332 (2000).
13. M. G. Mathieu et al., "HAGE, a cancer/testis antigen with potential for melanoma immunotherapy: identification of several MHC class I/II HAGE-derived immunogenic peptides," *Cancer Immunol. Immunother.* **56**(12), 1885–1895 (2007).
14. D. Ami, T. Neri, A. Natalello, P. mereghetti, S. M. Doglia, M. Zanoni, M. Zuccitti, S. Garagna, and C. A. Redi, "Embrionic stem cell differentiation studied by FT-IR spectroscopy," *Biochem. Biophys. Acta.* **1783**(1), 98–106 (2008).
15. G. J. Thomas, Jr., B. Prescott, and L. A. Day, "Structure similarity, difference and variability in the filamentous viruses fd, Ifl, IKe, Pf1 and Xf. Investigation by laser Raman spectroscopy," *J. Mol. Biol.* **165**(2), 321–356 (1983).
16. Z. Huang, H. Zeng, I. Hamzavi, D. I. McLean, and H. Lui, "Rapid near-infrared Raman spectroscopy system for real-time in vivo skin measurements," *Opt. Lett.* **26**(22), 1782–1784 (2001).
17. Z. Bacsó, L. Bene, L. Damjanovich, and S. Damjanovich, "IFN-gamma rearranges membrane topography of MHC-I and ICAM-1 in colon carcinoma cells," *Biochem. Biophys. Res. Commun.* **290**(2), 635–640 (2002).
18. F. Debierre-Grockiego, N. Molitor, R. T. Schwarz, and C. G. Lüder, "Toxoplasma gondii glycosylphosphatidylinositols up-regulate major histocompatibility complex (MHC) molecule expression on primary murine macrophages," *Innate Immun.* **15**(1), 25–32 (2009).
19. E. Carbone, G. Ruggiero, G. Terrazzano, C. Palomba, C. Manzo, S. Fontana, H. Spits, K. Kärre, and S. Zappacosta, "A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction," *J. Exp. Med.* **185**(12), 2053–2060 (1997).
20. G. Terrazzano, M. Sica, C. Gianfrani, G. Mazzarella, F. Maurano, B. De Giulio, S. De Saint-Mezard, D. Zanzi, L. Maiuri, M. Londei, B. Jabri, R. Troncone, S. Auricchio, S. Zappacosta, and E. Carbone, "Gliadin regulates the NK-dendritic cell cross-talk by HLA-E surface stabilization," *J. Immunol.* **179**(1), 372–381 (2007).
21. I. Notingher, "Raman spectroscopy cell-based biosensors," *Sensors* **7**, 1343–1358 (2007).
22. R. T. Maziarz, S. J. Mentzer, S. J. Burakoff, and D. V. Faller, "Distinct effects of interferon-gamma and MHC class I surface antigen levels on resistance of the K562 tumor cell line to natural killer-mediated lysis," *Cell. Immunol.* **30**(2), 329–338 (1990).
23. M. Nishimura, S. Mitsunaga, T. Akaza, Y. Mitomi, K. Tadokoro, and T. Juji, "Protection against natural killer cells by interferon-gamma treatment of K562 cells cannot be explained by augmented major histocompatibility complex class I expression," *Immunology* **83**(1), 75–80 (1994).
24. W. K. Chan, A. Sik-Yin Lau, J. Chun-Bong Li, H. Ka-Wai Law, Y. L. Lau, and G. Chi-Fung Chan, "MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-gamma challenge," *Exp. Hematol.* **36**(11), 1545–1555 (2008).
25. E. Hermann and H. Holger, "Cellular immunity to viral and fungal antigen after stem cell transplantation," *Curr. Opin. Hematol.* **9**(6), 485–489 (2002).