Technical Advance

Sampling Tumor-Draining Lymph Nodes for Phenotypic and Functional Analysis of Dendritic Cells and T Cells

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Immune responses against tumor antigens will initially occur in the first tumor-draining lymph node, the sentinel node (SN). Because of extensive diagnostic procedures, obtaining a piece of SN to isolate viable immune cells for functional analyses is often impossible. For this reason an alternative method to obtain viable cells from a lymph node (LN) was investigated, ie, scraping LNs with a surgical blade, and compared with dissociation of total LNs. Tumor-draining lymph nodes were retrieved from five oncological patients. The collected dendritic cells and T cells were phenotypically and functionally characterized by flow cytometry and antigen-specific interferon (IFN)- γ release in an ELISPOT assay. Results were compared between the two isolation methods. Viabilities and phenotypic characteristics of the collected cells were entirely comparable for both methods. T-cell functionality was also comparable between both methods, with equal T-cell expansion factors and similar frequencies of cytotoxic T cells specifically recognizing the M1 matrix protein of *Influenza haemophilus* or the tumor antigen *Her-2/neu*. In conclusion, scraping LNs to obtain cells for analysis of immune functions in LNs is feasible and presents a good alternative to dissociation of LNs. Scraping may even be applied to small LNs that a pathologist will submit entirely for histological examination and may thus prove useful in the monitoring of immune responses in SNs. (*Am J Pathol* 2002, 161:19–26)

Since the early 1990s a simple method has become available to detect and retrieve the first tumor-draining lymph node (TDLN), the so-called sentinel node (SN). This is done with a radioactive tracer and blue dye that are both injected around the tumor.^{1,2} During surgery, guided by the blue staining of the draining tissues, the radioactivity and the preoperatively made lymphoscintigraphy, the SN is removed and examined meticulously by the pathologist. Sections are cut and stained by hematoxylin and eosin and for specific tumor markers. If the sections from the center of the SN are tumor-negative, other sections at intervals of 250 μ m are cut and stained.³ Because of this extensive diagnostic processing, it is near impossible to obtain a fresh piece of the SN from which viable cells can be isolated.

Nevertheless, for immunological research, fresh SN material is important, because in these nodes the immune defense to tumor cells is initiated. The SN plays an important role in the interaction between a developing tumor and the host immune defense, sustaining the initiation and possibly even the maintenance of tumor immunity.^{4,5} In these processes, dendritic cells (DCs) play a very important role. They are uniquely equipped to activate naive T lymphocytes.^{6–9} To monitor tumor-specific cellular immune responses in TDLNs, specifically the SNs, obtaining viable cells from these nodes is of particular importance. For this reason we evaluated an alternative method in a relatively small pilot study to determine whether sufficient numbers of viable cells could be obtained from lymph nodes (LNs) for *in vitro* phenotypic and functional analyses so as not to interfere with diagnostic procedures.

DCs constitute an exceedingly small subpopulation of cells in LNs (<1%) and are therefore difficult to accurately characterize and quantitate.^{10–12} Usually, LNs are mechanically or enzymatically dissociated to obtain viable cells.^{13–17} According to Monfalcone and col-

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Table 1. Patient Characteristics, Location, and Size of the Removed LNs

Patient	Type of malignancy	Location of LN	Size of LN, mm
1	Pancreatic carcinoma	Mesenterium	18 × 14 × 5
2	Colon carcinoma	Mesenterium	4 × 4 × 3
3	Breast cancer	Axilla	5 × 4 × 4
4	Melanoma	Groin	8 × 5 × 5
5	Melanoma	Axilla	18 × 8 × 5

leagues,¹⁰ enzymatic dissociation of LNs is preferred above the mechanical dissociation technique. Dissociating the LNs enzymatically resulted in a large increase in cell yield, as well as in an increased cell viability. Especially relatively large and fragile cells, such as DCs, are shown in higher quantities by the use of the enzymatic dissociation technique.

Scraping fresh tissue with a surgical blade is often used in intraoperative settings, usually from tumors, to obtain cytological preparations for diagnostic procedures. This method is rapid, easy, and reliable.^{18–20} It is mainly used to detect malignant cells, but to our knowledge its usefulness for phenotypic and functional analysis of DCs and T cells has not yet been evaluated.

Because of the growing need for reliable immunological monitoring in SNs, we decided to evaluate the usefulness of this scraping method to sample the immunological status in LNs, focusing on DCs and T cells. We show that sampling a small piece of LN by scraping is a reliable alternative to total dissociation of the LN for immunomonitoring purposes.

Materials and Methods

Culture and Dissociation Media

Unless otherwise stated, all cultures were maintained in complete medium (CM) comprising Iscove's modified Dulbecco's medium (IMDM) supplemented with 25 mmol/L Hepes buffer (BioWhittaker, Verviers, Belgium) with 10% fetal calf serum, 50 IU/ml penicillin-streptomycin, 1.6 mmol/L L-glutamine, and 0.05 mmol/L 2-β-mercaptoethanol. For dissociation DNase/collagenase medium comprising RPMI 1640 (BioWhittaker) with 0.14% collagenase A (Boehringer Mannheim, Mannheim, Germany), 0.1% DNase I (Boehringer Mannheim), and 5% fetal calf serum was used.

Patients and LN Processing

From five oncological patients (Table 1) who underwent an elective LN dissection, one LN from the dissection specimen was used for this study according to institutional policies regarding the use of discarded tissue. Before surgery 40 to 50 ml of blood was drawn from each patient. Peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation using Lymphoprep (Nycomed Pharma AS, Asker, Norway). Cells were washed twice with sterile phosphate-buffered saline with 0.1% bovine serum albumin and

cryopreserved at –196°C in fetal calf serum containing 12.5% dimethyl sulfoxide.

All LNs were TDLNs, but not unequivocally identified as the SN. Directly after removal the LNs were collected in ice-cold CM. Further processing took place on a Petri dish containing 1 to 2 ml of CM on ice in a sterile environment.

First, fat was trimmed from the LN, after which it was bisected crosswise. From one cutting surface, imprint cytological specimens were made by dabbing the surface on an uncoated glass slide. The other cutting surface of the LN was scraped 10 times with a surgical scalpel blade (size no.22; Swann Morton Ltd., Sheffield, England) while holding the LN with a forceps. With the sharp, round edge of the blade cells were scraped off the surface of the LN. The blade was rinsed in DNase/collagenase medium.

The remains of the LN were cut into 2-mm³ pieces with a surgical scalpel followed by enzymatic digestion in DNase/collagenase. The specimens obtained by scraping and dissociation were collected in 15 to 30 ml of DNase/collagenase medium and kept in a water bath at 37°C for 45 minutes and were stirred meanwhile. After 45 minutes the cells were washed twice with 10 ml of CM and counted using a cytometer and Trypan-blue exclusion to determine yield and viability of the cells obtained by each of the isolation methods.

Immunocytochemical Staining of Imprint Specimens

Imprints from the cutting surfaces were made and air-dried overnight and fixed in acetone for 10 minutes. They were stained immunocytochemically using a biotin-streptavidin staining technique as described previously.²¹ Monoclonal antibodies (mAbs) recognizing CD1a, CD86 (both from Becton Dickinson, Mountain View, CA), and CD83 (Immunotech, Marseilles, France) were used at dilutions of 1:50, 1:10, and 1:10, respectively.

Flow Cytometry

Freshly isolated LN cells were directly stained with antibodies and analyzed by flow cytometry to establish their phenotypic characteristics. Single-color and dual-color analyses of cell surface markers were performed using combinations of mAbs labeled with either phycoerythrin or fluorescein isothiocyanate. Commercially available mAbs recognizing CD1a, CD4, CD8, CD86 (all from Becton Dickinson), and CD40 and CD83 (both from Immunotech) were used. The numbers of LN cells analyzed were 50 to 100,000 events per measurement.

T-Cell Expansion

After scraping or dissociation, T cells from the LN cells were expanded. Cells were incubated for 1 hour on ice with 2 μg of αCD3 per 1 × 10⁶ cells and 0.4 μg of αCD28 per 1 × 10⁶ cells (kindly provided by Dr. René van Lier,

CLB, Amsterdam, The Netherlands) in 100 to 200 μ l of CM with 5% fetal calf serum. After incubation the cells were placed on 24-well plates, coated with affinity-purified goat α -mouse immunoglobulin (1:100; DAKO, Glostrup, Denmark), in a concentration of 1×10^6 cells/ml/well for 1 hour at 4°C. The cells were cultured for 48 hours in a humidified 5% CO₂ incubator at 37°C. After 48 hours the cells were resuspended and each well was divided over four new uncoated wells, 250 μ l per well. To each new well 750 μ l of CM, containing interleukin-2 (CLB, Amsterdam, The Netherlands) at a concentration of 14 IU/ml, was added resulting in an end-concentration of ≥ 10 IU interleukin-2 per ml. The cells were cultured at 37°C in a humidified 5% CO₂ incubator for another 5 days. After 5 days the cells were harvested and counted. The described cycle was repeated once. After the second cycle the cells were harvested, counted, frozen, and stored for functional analysis at a later date. Expansion factors were determined.

Monocyte-Derived Dendritic Cell (MoDC) Culture

For functional analyses of expanded LN T cells, MoDCs were used as stimulator cells. The MoDCs were generated according to previously described methods.²² Briefly, plastic-adherent peripheral blood mononuclear cells were cultured for 7 days at a cell density of 3 to 5 $\times 10^6$ peripheral blood mononuclear cells per ml of CM, containing 100 ng/ml of granulocyte-macrophage colony-stimulating factor and 1000 IU/ml of interleukin-4. A typical phenotype (CD1a+/CD14-) and morphology was achieved.

Adenovirus Infection of MoDCs

After collection, the MoDCs were transfected with adenovirus encoding the M1 matrix protein of *I. haemophilus* (Adv-flu) (kindly provided by Dr. C Rickards and Dr. GW Wilkinson, University of Wales, Cardiff, UK) or adenovirus-green fluorescent protein (Adv-GFP) as a negative control. Transduction was CD40-targeted using a bispecific antibody conjugate as previously described by Tillman and colleagues.²³ Transduction of the MoDCs took place at a multiplicity of infection (MOI) of 100. The next day MoDCs were washed and seeded in precoated ELISPOT plates.

Peptide Loading of T2 Cells

For those patients who were HLA-A2-positive, T2 cells loaded with HLA-A2-restricted flu peptide (containing the M1-derived HLA-A2 binding epitope amino acids 58 to 66) or the HLA-A2-restricted HER-2/*neu* peptides (KIFGSLAFL and IISAVVGIL) were used as stimulator cells as well. As a control E7 peptide was used. T2 cells were pulsed overnight in serum-free CM with β -microglobulin (5 μ g/ml) and E7 peptide (50 μ g/ml), flu peptide (50 μ g/ml), or HER-2/*neu* peptide (50 μ g/ml) at 37°C in a

humidified 5% CO₂ incubator. The next day, cells were harvested and seeded in precoated ELISPOT plates.

Antigen-Specific IFN- γ Production by Memory CTLs

To analyze the expanded CD8⁺ T cells functionally, the IFN- γ ELISPOT assay was used.²⁴⁻²⁶ As effector cells, expanded α CD3/ α CD28-stimulated CD8⁺ T cells from the LN, obtained through dissociation or scraping, were used. Isolation of the expanded CD8⁺ LN lymphocytes was performed by using the untouched CD8⁺ mini MACS selection kit, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). After separation, cells were washed and resuspended in CM and added directly to the transduced MoDCs or loaded T2 cells.

The IFN- γ ELISPOT assay was performed as described previously.²⁷ Plates were seeded with 100 μ l of CM per well containing 5,000 stimulator cells (MoDCs or T2 cells) and 25,000 or 12,500 effector cells (CD8⁺ T cells), resulting in E/S ratios of 5:1 and 2.5:1, respectively. Each sample was tested in duplicate at each of the E/S ratios. Spots were counted by two independent observers using a microscope. To calculate the number of CD8⁺ T cells specifically responding to influenza, the mean numbers of spots induced by Adv-GFP-transfected MoDCs or E7 peptide-loaded T2 cells alone were subtracted from the mean spot numbers induced by Adv-flu-transfected MoDCs, flu peptide, or HER-2/*neu* peptide-loaded T2 cells. The specific response is expressed as the number of specific spots per 1,000,000 effector cells (ie, the number of influenza-specific or HER-2/*neu*-specific CD8⁺ T cells per 1,000,000 CD8⁺ T cells).

*HER-2/*neu*-HLA-A2-Tetramer-Binding Analysis*

Expanded and separated CD8⁺ T cells were stained with HLA-A2-restricted tetramers containing the HER-2/*neu* peptides KIFGSLAFL or IISAVVGIL. Phycoerythrin-conjugated HLA-A*0201 tetramer reagents were generated as described previously.^{28,29} Expanded and purified CD8⁺ T cells from peripheral blood lymphocytes (PBL) and obtained through dissociation and scraping were stained with the phycoerythrin-labeled tetramers (20 μ g/ml); incubation for 5 minutes in CM at 37°C in a humidified 5% CO₂ incubator, after which the cells were washed and kept at 4°C. The cells were analyzed by flow cytometry. The number of CD8⁺ T cells analyzed were at least 250,000 events per measurement.

Statistical Analysis

Data sets were compared nonparametrically using the paired Wilcoxon signed rank test. Correlation between CD4:CD8 ratios was determined using the nonparametrical Spearman test. $P < 0.05$ was considered significant.

Results

Yield and Viability of Collected LN Cells

Dissociation yielded an average of 53.0×10^6 (range, 7.0×10^6 to 1.9×10^8) cells. The mean yield of cells after scraping of the LN was 8.2×10^6 (range, 2.8×10^6 to 20×10^6) cells. Mean viability of the cells collected by scraping was 91% (range, 86 to 96%); mean viability of cells obtained by dissociation was 85% (range, 79 to 92%).

Phenotypic Analysis of DCs

Fluorescence-activated cell sorting (FACS) analyses were performed to determine the numbers, phenotypic characteristics, and activation state of lymph node dendritic cells (LNDCs). The membrane marker CD83 was used to detect mature DCs in the LN. CD1a and CD86 were used as additional DC and activation markers. A typical FACS analysis of LNDCs in a representative patient is shown in Figure 1A. In all but one patient (patient 4), the observed DC frequencies were lower among LN cells obtained through scraping compared to the LN cells obtained after dissociation. However, the paired non-parametrical Wilcoxon test revealed no significant differences between the two methods (Figure 1B). All CD83⁺ cells were positive for CD86 as well. In two patients (patients 1 and 2) no CD1a-positive cells were detectable. The LNs from these two patients were located in the mesenterium, whereas the LNs from the other three patients were skin-draining LNs (Table 1).

The marker expression levels of the DC markers CD83, CD1a, and CD86 were comparable between both methods. ($P = 0.686$, $P = 0.138$, and $P = 0.500$) (Figure 1B).

The FACS results were consistent with the immunocytochemical imprints made from the tested LNs. Staining for CD1a, CD83, and CD86 showed numerous strongly positive cells with typical DC morphology (Figure 1C). Imprints made from the mesenteric LNs in patients 1 and 2, showed no CD1a-positive cells.

Phenotypic Analysis of T Cells

The CD4 or CD8 phenotype of LN T cells was determined by flow cytometry. T cells were expressed as CD4:CD8 ratios for each method. CD4:CD8 ratios of collected T cells varied between patients, but within patients there was a significant correlation (correlation coefficient, 0.94) between CD4:CD8 ratios found immediately after dissociation or scraping ($P = 0.037$, Figure 2). Mean percentages of CD4⁺ T cells obtained through dissociation or scraping were 32.82% (range, 28.12 to 38.63%) and 36.67% (range, 25.95 to 50.55%) ($P = 0.345$), respectively. For CD8⁺ T cells, these mean percentages were 9.86% (range, 6.18 to 12.46%) and 9.55% (range, 5.06 to 11.62%) ($P = 0.500$), respectively.

T-Cell Functional Analysis

To obtain sufficient numbers of CD8⁺ T cells for functional analyses, LN T cells were nonspecifically stimulated and expanded. T-cell expansion factors after α CD3/ α CD28 stimulation were similar for the dissociation method and the scraping method, 52.7 (range, 6.3 to 97.9) and 51.2 (range, 6.2 to 102.0) ($P = 0.786$), respectively.

After expansion functional analyses of separated and purified CD8⁺ T cells were done. As model antigen, the M1 matrix protein of *I. haemophilus* (Adv-flu) and an M1-derived HLA-A2-binding epitope amino acids 58 to 66 (flu peptide), were used. Flu-specific CD8⁺ T-cell frequencies were detected using Adv-flu-transduced MoDCs or flu peptide-loaded T2 cells, without previous *in vitro* restimulation.

Although differences existed between all patients in the frequency of detectable IFN- γ -secreting influenza-specific CD8⁺ T cells using the Adv-flu-transduced MoDCs, in individual patients the frequency of influenza-specific CD8⁺ T cells (ie, the difference between the spots induced by Adv-flu-transduced MoDCs and the number of spots induced by GFP-transduced MoDCs) among CTL obtained by dissociation or scraping were comparable ($P = 0.786$, Figure 3A). In contrast, using the flu peptide-loaded T2 cells in the HLA-A2-positive patients 2 to 5, some minor discrepancies were found in frequencies of epitope-specific T cells obtained by dissociation or through scraping (for patients 3 and 5, Figure 3B). In general, frequencies of CD8⁺ T cells specifically recognizing the HLA-A2-binding flu-epitope were lower as compared to CTL responding to the entire M1 sequence.

Analyses of HER-2/neu-Specific CTL in a HLA-A2-Positive Patient with HER-2/neu-Positive Primary Breast Cancer

One of the tested patients (patient 3) was found to be HLA-A2-positive and to carry a HER-2/neu-positive breast tumor. This offered the possibility to test the utility of both isolation methods to detect tumor-specific CTL, using HER-2/neu as model tumor antigen.

Higher detectable frequencies of IISAVVGIL-specific CD8⁺ T cells were found with both isolation methods by tetramer binding in phenotypic analyses, compared to KIFGSLAFL-specific CD8⁺ T cells (Figure 4A). This was in accordance with the results found by the ELISPOT assay in the functional analyses (Figure 4B).

In comparison, in the expanded PBL very low frequencies of IISAVVGIL-specific CD8⁺ T cells were found, consistent with the idea that immunological responses against tumor antigens will preferentially occur in TDLNs (Figure 4A).

Discussion

In this study we have shown that isolation of viable cells from a LN by scraping with a surgical scalpel to sample local immunological responses, is a very suitable alter-

Figure 1. A: FACS analyses of LN cells obtained through scraping the cutting surface of TDLNs with a surgical scalpel in a representative patient (patient 3). CD83/iso, CD83/CD1a, and CD83/CD86 staining of axillary LN cells. **B:** FACS analysis data for LNDCs per patient. LNDC frequencies are expressed as the percentage of total LN cells and LNDC marker expression levels are expressed as mean fluorescence (logarithmic scale) for the dissociation method and the scraping method. The mean percentages are the percentages of the total number of counted LN cells. Mean DC frequencies for CD83⁺, CD83⁺/CD1a⁺, and CD83⁺/CD86⁺ obtained with the dissociation method were 0.51% (range, 0.33 to 0.85%), 0.28% (range, 0.01 to 0.75%), and 0.55% (range, 0.17 to 1.31%), respectively. With the scraping method these frequencies were 0.42% (range, 0.05 to 0.74%), 0.25% (range, 0.01 to 0.73%), and 0.44% (range, 0.15 to 0.93%), respectively. The mean expression levels of the DC markers CD83, CD1a, and CD86* for the dissociation and scraping method were 77 versus 77, 1365 versus 1706, and 163 versus 128, respectively. Paired Wilcoxon signed rank test used between methods revealed no significant differences. *, Double-positive for CD83. **C:** Immunocytochemical stainings for the DC markers CD1a, CD83, and CD86 on imprint specimens of an axillary LN from a breast cancer patient (patient 3). Original magnifications, ×400.

native for dissociation of LN, which can be considered the gold standard to isolate viable LN cells.

DCs and T cells in TDLNs are crucial in the immune response against tumor antigens. In the first-line TDLNs,

ie, SN, the first immunological response against tumor antigens will occur.^{6,8,9} Immune functions in TDLNs are often strikingly down-regulated.^{30–32} Monitoring and making an inventory of immune responses in SN is thus of

Figure 2. Correlation of CD4:CD8 ratios among T cells isolated from LN by dissociation or scraping. Spearman's nonparametric correlation test; correlation coefficient of 0.94, $P = 0.037$.

Figure 3. Specific frequencies of influenza M1 matrix protein-specific CD8⁺ T cells. **A:** Adenovirus M1-transduced monocyte-derived dendritic cells were used as stimulator cells (paired Wilcoxon signed rank test between methods, $P = 0.786$). All assays were done in duplicate, except for patient 1. **B:** Specific frequencies of influenza peptide (M1-derived HLA-A2-binding epitope amino acids 58 to 66)-specific CD8⁺ T cells in HLA-A2-positive patients (patients 2 to 5). T2 cells loaded with flu peptide were used as stimulator cells (paired Wilcoxon signed rank test between methods, $P = 0.715$). All assays were done in duplicate. Number of influenza-specific spots (ie, influenza-specific CD8⁺ T cells) per 1,000,000 expanded LN CD8⁺ T cells are shown (■, dissociation; □, scrape).

Figure 4. **A:** FACS analyses with HER-2/*neu* tetramers for the epitopes KIFGSLAFL and IISAVVGIL of isolated CD8⁺ T cells derived from PBL, dissociated LN, and scraped LN material from a HLA-A2-positive patient (patient 3) with proven HER-2/*neu*-positive breast cancer. **B:** IFN- γ Elispot data from the same patient: T2 cells were used as stimulator cells, loaded with HER-2/*neu* epitopes KIFGSLAFL and IISAVVGIL. Responder cells were CD8⁺ T cells from dissociated or scraped LN material (■, dissociation; □, scrape). Specific frequency determined at an effector/stimulator ratio of 1.25:1 (10,000 loaded T2 cells). Each sample was tested in duplicate.

particular importance. For this, isolation of viable DCs and T cells from the SN is important to test the functional status of these immune effector cells.

Viable LN cells are usually obtained through dissociation of the LN. Because of extensive diagnostic processing of SNs, obtaining even a small piece of these nodes for dissociation is impossible.³ For this reason, we compared this dissociation method with an alternative method in which cells were obtained through scraping of LN. These two methods were previously compared for tumor tissue.³³ Torres and colleagues³³ compared dissociation of breast adenocarcinoma with scraping of the tumor and concluded that the methods were comparable.

As was to be expected, the absolute cell yield was higher after enzymatic dissociation of the entire LN, but the cell yield from scraping a cutting surface of the LN was well above the number of cells considered adequate for flow cytometric analyses and T cell expansion. A cell yield in the range of 1.5×10^6 to 2.5×10^6 was sufficient for the analyses described in this study, which were realized for all of the patients studied. Viability of LN cells obtained through scraping was at least as high and often higher than that of cells obtained through dissociation. Although the number of cells obtained after scraping seemed to be rather high in relation to the total yield after dissociation, in our experience dissociation is a relatively

inefficient method resulting in an underrepresentation of the total number of cells actually present in a LN. Moreover, the total volume of tissue collected by scraping is negligible in comparison to the total volume of the LN and interferes in no way with standard histopathological diagnostic procedures.

Most importantly, no phenotypic or functional skewing through scraping was observed as compared to dissociation. In phenotypic analyses, although in lower amounts, we were able to detect DCs after scraping in relatively similar quantities as after dissociation. A possible reason for detecting slightly less DCs after scraping could be the fact that DCs are large, fragile cells that can easily be damaged using mechanical processing.¹⁰ DC marker expression levels were comparable between both methods, suggesting that no maturation of DCs occurred because of mechanical manipulation associated with the scraping method.

To assess morphological aspects of DCs, imprint cytology of LNs proved to be very suitable. Cells with morphological DC characteristics stained positive for CD1a, CD83, and CD86 in imprints, thus confirming our FACS data to be DC-specific. Interestingly, DCs obtained from mesenteric LNs (patients 1 and 2) did not stain positive for CD1a. Takahashi and colleagues^{12,34} also reported that CD1a⁺ DCs are present in superficial LNs, but not in deeply located LNs, suggesting that DCs in mesenteric LNs are derived from CD1a⁺ DC precursors, of which the nature and origin remains unclear.

T cells were obtained by both isolation methods in equal quantities. There was a near exact correlation between CD4:CD8 ratios of T cells obtained through dissociation and scraping. In the antigen-specific functional analyses of CD8⁺ T cells with transduced MoDCs, we used the entire sequence of the influenza antigen, with all possible epitopes, to stimulate the CTLs. In these analyses, the frequencies of IFN- γ producing flu-specific CD8⁺ T cells were equal for both isolation methods. Using T2 cells loaded with flu peptides gave slightly different results between the two isolation methods in two patients. A possible explanation for this may be that in the scraping method only one specific level of the LN is sampled, whereas in the dissociation method the whole LN or a larger part of the LN is sampled. It is conceivable that at the specific level of sampling through scraping, a T-cell clone with particular epitope recognition is located, in which case a relatively larger number of CD8⁺ T cells specifically recognizing this epitope will be detected after scraping, compared to the dissociation method, or vice versa.

Furthermore, this study demonstrated the feasibility of detecting low frequent tumor-specific CD8⁺ T cells in expanded T cells obtained through scraping LNs. The frequencies of Her-2/*neu*-specific CD8⁺ T cells detected after scraping were comparable with those obtained through dissociation. This was confirmed using two different detection methods: MHC-I-binding tetramers and the IFN- γ ELISPOT assay. Significantly lower frequencies of Her-2/*neu*-specific CD8⁺ T cells were detected in corresponding PBL samples. This is consistent with the

idea that immunological responses against tumor antigens will preferentially occur in TDLNs.²⁵

The scraping of LNs has several advantages over total dissociation of the specimens, in that the LN is not consumed in the dissociation process and can be further processed for routine histological examination. It might also be applied in vaccination studies or studies in which patients are treated with immunostimulating adjuvants (eg, granulocyte-macrophage colony-stimulating factor), to monitor T-cell responses in local LNs. Furthermore, scraping is faster and more convenient than dissociation of LNs. However, it should always be performed by or in coordination with the pathologist so that definitive histological examination of these LNs is not compromised.

In conclusion, scraping LNs to obtain cells for functional and phenotypic analysis to determine the immunological responses in LNs is feasible and seems to be comparable with cells obtained through dissociation of LNs. Scraping may therefore be the solution to collect viable LN cells from TDLNs and specifically SN for phenotypic and functional analysis without compromising the diagnostic examination of these LNs by pathologists.

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