Clin. Lab. Haem.
1997, **19**, 231–241

Guidelines for the enumeration of CD4⁺ T lymphocytes in immunosuppressed individuals

CD4⁺ T Lymphocyte Working Party
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Keywords CD4, T-lymphocytes, HIV, AIDS, flow cytometry

Introduction

The acquired immunodeficiency syndrome (AIDS) results from a severe and progressive functional loss of CD4⁺ T-lymphocytes. The aetiological agent is the human immunodeficiency virus (HIV) which causes a progressive depletion of CD4⁺ T-lymphocytes, together with activation and dysfunction of many other cells involved in the immune response. The resulting clinical immunodeficiency is manifest by the occurrence of opportunistic infections, selective increase in specific tumours, frequent association with wasting and potential involvement of the central nervous system. Because of the association of disease progression with the decline in CD4⁺ T-lymphocytes, the management of HIV infected individuals requires the precise and accurate measurement of this lymphocyte subset (Centers for Disease Control 1992; Nicholson 1994). CD4⁺ T-lymphocyte quantitation is determined by flow cytometric immunophenotyping of peripheral whole blood and is essentially based upon a product of three laboratory variables: the white blood cell count (WBC), the lymphocyte count and the percentage of lymphocytes that express the CD4 antigen. Flow cytometric immunophenotyping facilitates the identification of individual cells according to size, granularity and antigen expression. The reliance of analysis upon these parameters, and the move of flow cytometry from research to routine clinical practice, has increased the need for standardization. To ensure the accuracy and reliability of CD4⁺ T-lymphocyte counting, on both an intra- and inter-laboratory basis, standard methods as well as guidelines for quality assurance and quality control are required. This guideline provides information on: (1) frequency of CD4 testing, (2) laboratory safety, (3) specimen collection, transport and storage, (4) specimen processing and controls, (5) instrument quality control, (6) sample analysis, (7) data analysis, storage and reporting and, (8) quality assurance.

Accepted for publication 18 July 1997
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Frequency of CD4 testing

The frequency of monitoring a patient's CD4 count will depend upon a number of factors, including the treatment and the rate of disease progression. All HIV⁺ patients should have at least one baseline CD4 measurement at the time of diagnosis and then monthly for 3 months following HIV diagnosis. Following this, asymptomatic patients will require CD4 counts every 6 months, while symptomatic patients require analysis at 3 monthly intervals. Individuals with rapidly decreasing levels, as detected on the first three measurements, should have a CD4⁺ T-lymphocyte count checked monthly. The CD4⁺ T-lymphocyte count should be used as a guide for the administration of antiretroviral therapy. *Mycobacterium avium-intracellulare* (MAI) prophylaxis, and *Pneumocystis carinii* pneumonia (PCP) prophylaxis in adults. The threshold for antiviral/MAI therapy is a CD4 count $< 0.5 \times 10^9$/l; for PCP prophylaxis a CD4 count $< 0.2 \times 10^9$/l.

Laboratory safety

Each individual laboratory must have local guidelines describing the procedures involved in the laboratory handling of pathological specimens. The Advisory Committee on Dangerous Pathogens has recently reviewed and published workplace guidelines which provide information on the categorization of, and protection against, blood-borne infections such as HIV and hepatitis (Advisory Committee on Dangerous Pathogens, 1995). All laboratory workers must

be familiar with these guidelines before undertaking such work.

The decontamination of the flow cytometer, following the analysis of HIV specimens, must be performed in accordance with the manufacturer's recommended instructions. Stream-in-air flow cytometers should not be used for $CD4^+$ T-lymphocyte counting on HIV infected material. If this is not possible, measures to avoid aerosols or droplets of sample material must be undertaken (if in doubt contact the manufacturer of the instrument). The use of a flow cytometer equipped with an auto-sample loader/bio-sampler to minimize sample handling is recommended.

Sample preparation should be performed in a designated area, which should be thoroughly decontaminated after use. Test-tubes should be individually capped in order to prevent spillage and to retain aerosols that may be created during mixing. Spillages of HIV-infected material should be dealt with immediately by using an appropriate decontaminating solution in accordance with health and safety guidelines. After staining, lysing and washing, buffered paraformaldehyde (pH 7.0–7.4, 2% solution) or a proprietary cell-fixing reagent, should be added to the samples in order to minimize the infection risk. All samples must remain in fixative after analysis. Flow cytometer systems that employ a sample loader/bio-sampler may use a 'lyse-no-wash' procedure in order to minimize handling. In these instances, fixation of the specimen may not be required prior to analysis, providing the loader is sealed when in use. However, following analysis, all samples should be inactivated prior to disposal, using either a 10% solution of sodium hypochlorite, a viral inactivating reagent, or ideally a combined viral inactivating and mycobacteriocidal reagent. The sample-loader/bio-sampler should be thoroughly decontaminated after use in accordance with the manufacturer's guidelines. Specimens should be disposed of by appropriate methods.

Specimen collection

All specimens from individual patients should, wherever possible, be collected by venepuncture at the same time of day to minimize diurnal variation which can produce significant changes in absolute CD4 counts. It is important that the correct anticoagulant is used with 0.34 M di- or tri-potassium ethylenediamine tetra-acetic acid (K_2EDTA or K_3EDTA) being preferred. *Haematological analysis must be completed within 6 h of venepuncture.* If a specimen is to be referred to a central laboratory for analysis, resulting in a delay of over 6 h, then a total WBC must accompany the sample (it is preferable that the total WBC and flow cytometric differential, rather than a haematological analyser derived absolute lymphocyte count, be used to calculate the absolute CD4 lymphocyte count). *All samples must be labelled with a unique patient identifier and data and time of collection and be fully processed within 18 h to minimize anticoagulant effect. The use of soundex or hospital reference numbers to ensure confidentiality is preferred. The possible risk of infection should be clearly indicated on sample(s) and laboratory request form(s).*

Storage and transportation must be between 10°C and 30°C and samples should not be subjected to temperatures below 10°C (Ekong *et al.* 1992). Packaging and transportation of HIV infected material should be in accordance with the regulations of the postal and/or courier service used. Specimen integrity must be examined upon receipt and repeat specimens requested if there is evidence of gross haemolysis or clots or if the specimen is received >18 h from time of venepuncture.

Specimen processing and controls

There are several methods of specimen processing and determination of absolute T lymphocyte subsets, all of which involve the staining of lymphocytes in whole blood with monoclonal antibodies directly conjugated with fluorochromes. The lymphocyte population is analysed by flow cytometry and the absolute lymphocyte subset values calculated accordingly.

Two colour flow cytometry and ultra-violet microscopy

In the early 1980s determination of $CD4^+$ T lymphocytes was usually undertaken using Ficoll-density-separated lymphocytes and ultra-violet (UV) microscopy. *It must be stressed that enumeration of $CD4^+$ T lymphocytes should no longer be undertaken by either UV microscopy or single colour analysis.* There have been a number of technological advances that have enabled the routine use of whole blood lysis with multi-colour parametric analysis. In the mid to late 1980s, changes in software and conjugation of new fluorochromes to monoclonal antibodies facilitated identification of lymphocytes, using either light scatter gating procedures or differential staining with CD45 and CD14 (Loken *et al.* 1990). These approaches, however, have several disadvantages. Firstly, a forward/side scatter gate approach does not enable the identification of gate contaminants and may result in falsely low percentage values, while the requirement for larger panels (up to six tubes in a panel) increases analysis time, specimen handling and ultimately cost. Secondly, it is impossible to detect tube-to-tube variation when using a light scatter gate derived from

CD45/CD14 'back-gating'. In addition, the isotype control tube does not provide a control for CD45/CD14 staining, while the light scatter gate may contain granulocytes and/or red cell debris necessitating result correction (basophils cannot be resolved from the lymphocyte population based upon a light scatter gate using CD45/CD14).

In view of the above limitations the optimal method for T lymphocyte evaluation is now multi-parametric or three colour lymphocyte immunophenotyping. This approach has substantial advantages as it enables analysis of high purity lymphocyte gates with low levels of debris and/or contaminating cells. *Thus it is recommended that CD4+ T lymphocyte determination is performed using a minimum of three colour lymphocyte immunophenotyping employing one of the following three gating strategies: (1) T-gating, (2) lineage gating and (3) CD45/side scatter (SSC) gating.* Each of these approaches will be described in more detail below.

T-Gate method

This approach, using whole blood lysis, is best suited to flow cytometers capable of generating absolute lymphocyte counts (Mandy *et al.* 1992). The technique can be used on instruments without such facilities but will complicate the reporting of both absolute lymphocyte counts and percentages (note: that the light scatter profile [forward angle (FSC) *v.* right angle/side scatter (SSC)] is not used as the primary gate). The absolute numbers of T-lymphocyte subsets can be derived from a single tube containing a mixture of anti-CD3, anti-CD4 and anti-CD8, conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) and a third fluorochrome (e.g. peridin chlorophyll protein [PerCP]) respectively (Table 1). Isotype controls are not necessary. Following staining, lysis and washing, the specimen is analysed using a combination of right angle light scatter and the CD3 fluorescence channel. This allows identification of the T lymphocyte cluster and the setting of the relevant gate (Figure 1). Events are collected then analysed using the remaining fluorescence channels, i.e. FL2 VFL3 (Figure 2). The events collected through this gate are, by definition, T lymphocytes (i.e. expressing CD3), allowing the identification of CD4 and CD8 positive populations. This method of analysis excludes B-lymphocytes, $CD8^+$ NK cells, monocytes and debris. The $CD4^+$ and $CD8^+$ lymphocytes, therefore, are expressed as a percentage of the total $CD3^+$ lymphocytes and not the total lymphocyte population. The advantages and disadvantages of the T-gating method are listed in Table 2. Advantages include: (i) a large T-gate may be used to maximize recovery without sacrificing purity; (ii) the identification of dual positive populations ($CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$) are determined in a single tube; (iii) immature and $\gamma\delta$ T-cells are not

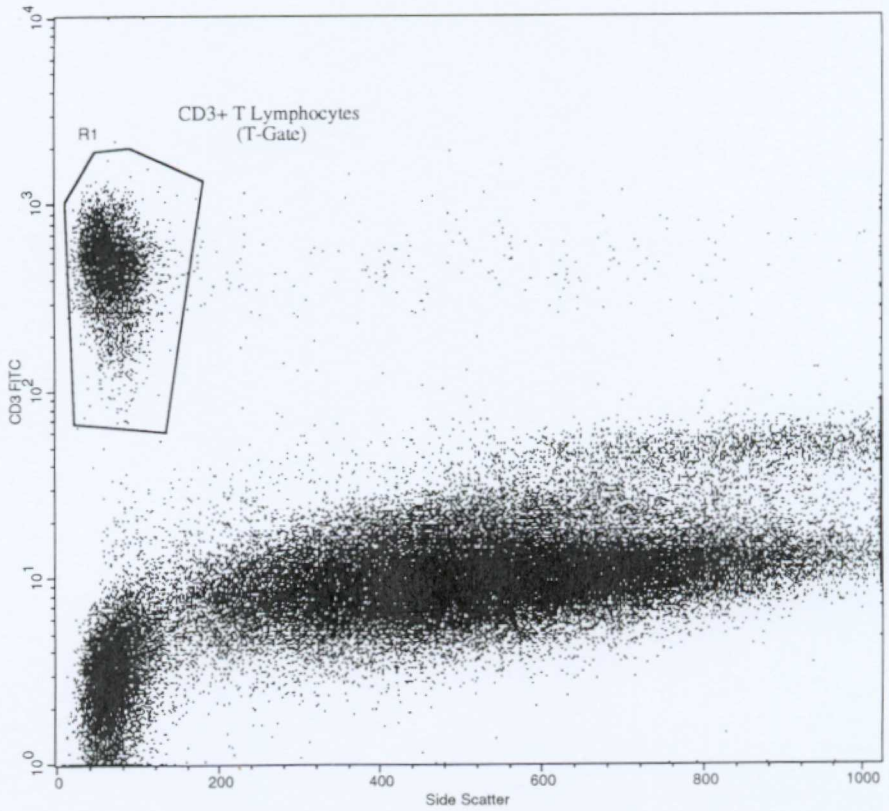

Figure 1. T-Gate method (CD3/SSC) dot plot of a whole blood lysis specimen. R1 denotes the $CD3^+$ T lymphocyte analysis region.

T-Gating Fluorochrome order e.g. (FITC/PE/PE-Cy5)	Lineage Gating Fluorochrome order e.g. (FITC/PE/PE-Cy5)	CD45/SSC Gating Fluorochrome order e.g. (FITC/PE/PE-Cy5)	Tube
CD3/CD4/CD8	Isotype controls e.g. (IgG_1/IgG_{2a}/IgG_1)	IgG_1/IgG_1/CD45 (this tube is optional)	No. 1
	CD3/CD4/CD8	CD3/CD19/CD45	No. 2
	CD16 &/or CD56/CD19/CD3	CD3/CD4/CD45	No. 3
		CD3/CD8/CD45	No. 4
		CD3/CD16 &/or CD56/CD45	No. 5

Table 1. Monoclonal antibody panels required for T-gating, lineage gating and CD45/SSC gating (these are examples only, exact combinations and/or fluorochrome assignment will depend upon instrument configuration and software analysis/gating procedure used)

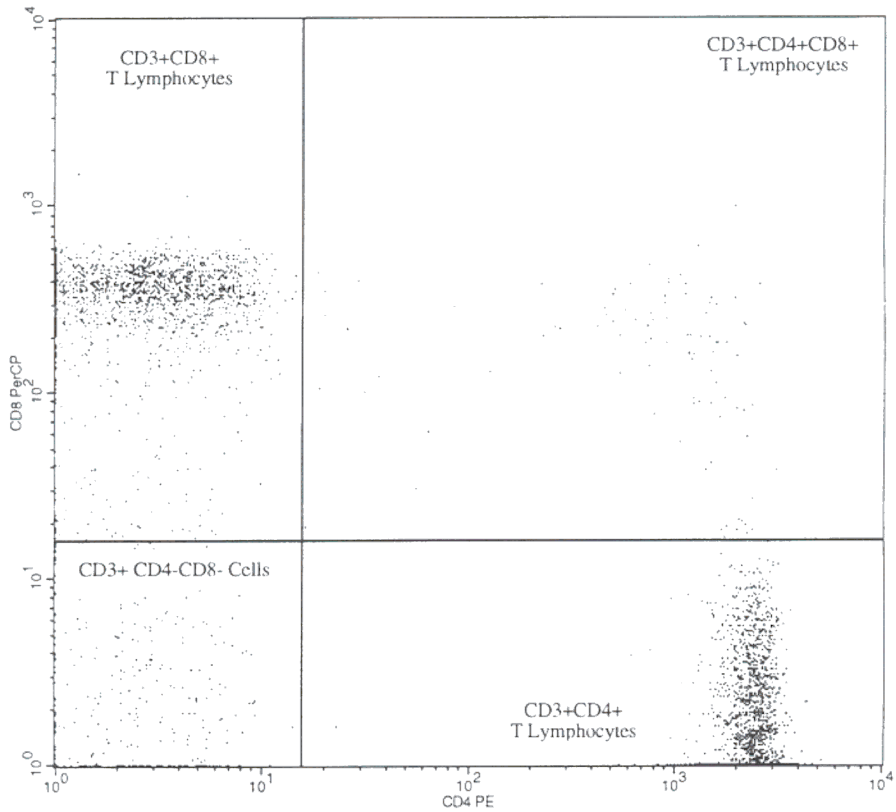

Figure 2. Analysis of $CD3^+$ T lymphocytes identified in Figure 1 (R1) for expression of CD4 and CD8.

included in the $CD4^+$ and $CD8^+$ determinations and (iv) an isotype control is not required. However, these advantages should also be considered with the disadvantages which include: (i) the exclusion of B-lymphocytes and NK-cells results in the lack of a full lymphocyte immunophenotypic profile; (ii) $CD4^+$ and $CD8^+$ lymphocyte populations are expressed as a percentage of $CD3^+$ lymphocytes and not of the total lymphocyte population; (iii) use of a single tube assay does enable the calculation of the lymphosum (total T cells (CD3%)+B cells (CD19%)+NK-cells ($CD3^-$CD16 and/or $CD56^+$%) = 100% ± 5%) nor does it enable replicate comparisons of $CD3^+$ cells, which are required for quality control purposes; (iv) absolute values of CD4 and CD8 positive cells cannot be determined if the flow cytometer has no direct counting facility, and (v) the T sum (CD4+CD8 = CD3) may not necessarily be helpful in HIV cases where $CD3^+CD4^-$ $CD8^-$ cells are present. The role of this technique, therefore, is best suited to instruments, or reagent systems, that provide direct absolute counts, using either beads or precision delivery.

Lineage gating

A logical development of the T-gating method is the inclusion of B lymphocytes and NK-cells. This was first

Table 2. Advantages and disadvantages of using T-lineage gating procedures

T-Gate method advantages	T-Gate method disadvantages
A large T-gate can be used to maximize recovery	B & NK lymphocytes, vital for lymphosum, are excluded
A pure T-gate is obtained thus avoiding result correction	$CD4^+$ & $CD8^+$ populations are expressed as a percentage of $CD3^+$ lymphocytes
Identification of dual positive CD4 and CD8 populations is possible	Use of a single tube does not provide lymphosum or allow comparative CD3 determinations for internal QA
Immature and $\gamma\delta$ T-cells are not included	Absolute values for CD4 & CD8 cannot be determined without using a flow cytometer with absolute counting capability
Isotype controls are not required	The T sum (CD4+CD8 = CD3) may not be helpful in cases when $CD3^+CD4^-CD8^-$ cells present
A one tube panel is used	Are best suited to instruments with absolute count capability
Cost is lower than a conventional larger two-colour panel	
There are fewer tubes to handle and process than with a two-colour panel	
It is suitable for both paediatric and adult specimens	
There is an increased number of evaluable samples when compared with the use of FSC/SSC gating strategies	

proposed by Mercolino *et al.* (1995) and is referred to as 'lineage gating'. The procedure encompasses T-lineage as well as B-lineage gating strategies so as to include all the lymphocyte subsets. As a result, using three tubes (including isotype controls), it is possible to analyse T-, B- and NK lymphocytes, as well as obtaining duplicate T lymphocyte analysis and lymphosum enumeration. The latter two procedures are required for internal quality control purposes. The panel of tubes for lineage gating are detailed in Table 1.

Lineage gating analysis incorporates the whole blood lysis technique. An isotype control tube is used to set a light scatter gate (FSC *v* SSC) on the lymphocyte population. Events collected within this gate are then used to determine the negative and positive regions on the three fluorescence channels. The use of the control tube also enables the identification of non-specific staining in the SSC versus fluorescence gates used in the analysis of specific cell populations. The second tube, containing the fluorochrome conjugated CD3, CD4 and CD8 antibodies, is used to analyse T lymphocytes in the manner described for T-gating. The third tube, containing an NK marker (i.e. anti-CD16), a B lymphocyte marker (i.e. anti-CD19) and the T lymphocyte marker anti-CD3, enables the calculation of the lymphosum as well as allowing a duplicate check of $CD3^+$ T lymphocytes. Several gates are used to achieve these values, including light scatter gates, T-, B- and NK-gates (see Mercolino *et al.* [1995] for an in depth description of the procedure). In addition to the advantages of T-gating, the technique enables: (i) the exclusion of debris and non-lymphoid cells from analysis, thus increasing the recovery without compromising on purity, and negating the requirement for flow cytometric values to be corrected for non-lymphocyte contamination; (ii) a complete lymphocyte profile from three tubes (including controls) is obtained, making it suitable for small volume samples; (iii) a lymphosum can be obtained, thus providing an internal quality control check; (iv) the T-lymphocyte subsets to be reported as a percentage of the total lymphocytes and; (v) the resolution of the basophils from the lymphocyte population. The disadvantages of this approach are: (i) that it can only be used on flow cytometers with absolute count facilities; (ii) that it is dependent on advanced software and gating techniques; (iii) that the use of SSC is the primary method of discriminating lymphocytes from granulocytes and also; (iv) that difficulties may be encountered in NK cell gating if a single marker is used for their identification due to the heterogeneity of antigen expression by NK cells.

CD45/SSC gating

It is well recognized that relying solely upon light scatter gating may lead to problems with contamination. As a result, methods have been devised to optimize the placement of light scatter gates and, at the same time, provide information of gate contamination by non-lymphocytes and debris. The use of a tube, containing both anti-CD45 and anti-CD14, was initially proposed by Loken *et al.* (1990) to overcome such problems and is the basis of the Centers for Disease Control (CDC) recommendations for $CD4^+$ T lymphocyte immunophenotyping (Centers for Disease Control 1992; Centers for Disease Control 1993). However, several problems still exist with this approach.

Firstly, the tube containing anti-CD45 and anti-CD14 is not subjected to isotype control monitoring. Secondly, the gate set, using this combination, is assumed to be constant throughout the panel used and finally the remaining panel uses a light scatter gate making discrimination between debris and lymphocytes difficult and discrimination between basophils and lymphocytes impossible. Nicholson, Hubbard & Jones (1996) therefore, proposed the use of a three colour procedure, incorporating anti-CD45, which is used to provide an immunological tube-to-tube check of the lymphocyte analysis region. The approach requires that anti-CD45 is incorporated into each combination of antibodies (see Table 1). As with T- and lineage gating, the approach uses fluorescence versus side scatter. CD45 expression is used to identify the lymphocyte population ($CD45^{bright}$, low linear SSC) (Figure 3). The lymphocyte gate thus defined is relatively free from contaminating cells and debris. The remaining two or more fluorescence channels are then analysed through this region (Figure 4). Because there are very few contaminating events, the need to correct for non-lymphocyte contamination is reduced and the accuracy of lymphocyte determination increased. For a more in-depth description of this procedure see Nicholson *et al.* (1996). As with the T-gating method, isotype controls are not required, the remaining unstained cells are used to determine the negative and positive fluorescence boundaries. Table 1 details the antibody combinations which should be used to enable immunophenotyping of HIV infected individuals by this method.

There are several advantages to using CD45 gating: (i) it can be used with instruments and reagent systems that do not provide absolute counts; (ii) the use of anti-CD45 in each tube allows consistent gating checks on an inter-tube basis; (iii) no debris is collected in the analysis gate; (iv) non-lymphocyte contamination is minimal; (v) it discriminates basophils from lymphocytes (not possible by CD45/CD14 gating); (vi) high purity lymphocyte data is collected which avoids the need for result correction; (vii) replicate CD3 determinations are obtained while the $CD3^+CD4^+$ and $CD3^+CD8^+$ populations are determined in only two tubes; (viii) cost may be lower than with a larger, two-colour panel; (ix) no isotype controls are required; (x) there are fewer tubes to handle and process and; (xi) there are increased numbers of evaluable samples compared to FSC/SSC gating strategies. However, there are also several disadvantages which need to be considered: (i) added expertise is required for machine set-up (including spectral compensation), data collection and data analysis; (ii) the third fluorochrome may not be detected by older flow cytometers; (iii) CD4 and CD8 determination is performed in separate tubes; (iv) some lymphocytes may be lost from analysis due to weak CD45 expression and; (v)

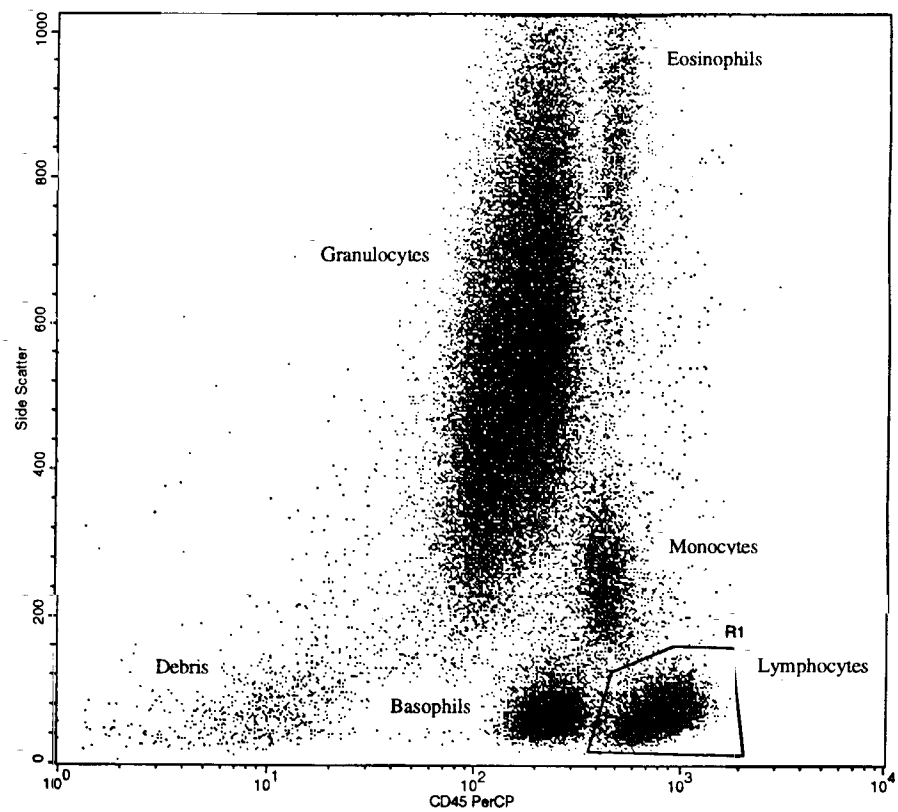

Figure 3. CD45/side scatter (SSC) dot plot of a whole blood lysis specimen. The relative position of each leucocyte population is shown. R1 denotes the lymphocyte analysis region. (Data collected on a Becton Dickinson FACScan using Cell Quest software™.)

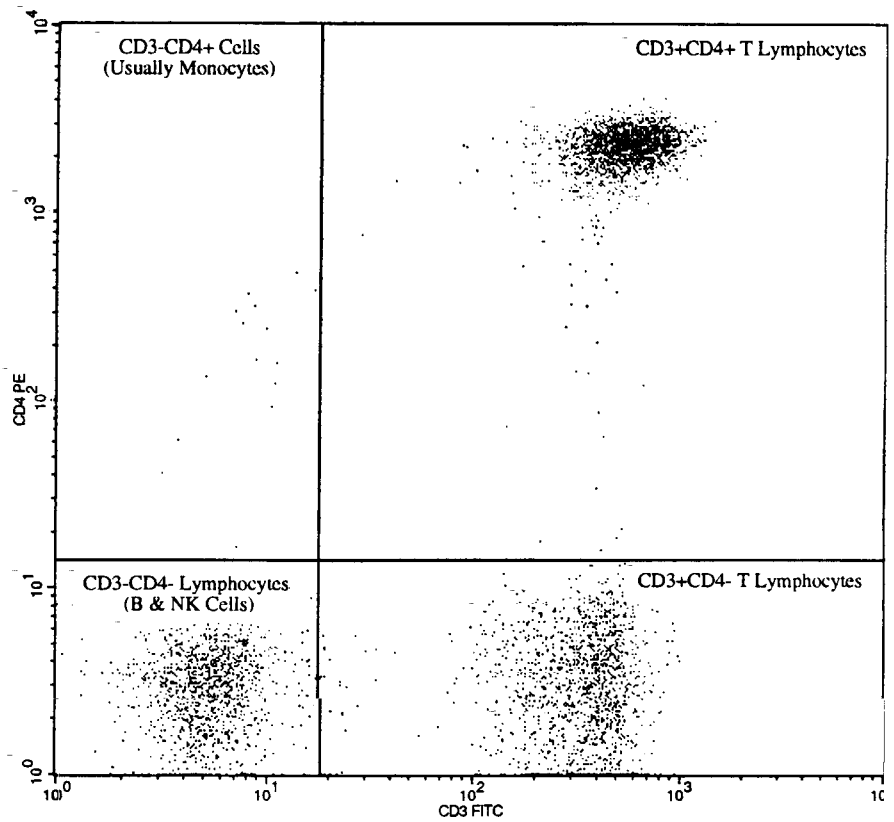

Figure 4. Analysis of CD4+ T lymphocytes using the R1 region designated in Figure 3.

discrimination of lymphocytes from smaller monocytes may be difficult in some cases.

To overcome the limitations of three colour immunophenotyping, four colour technology is now becoming available, incorporating CD45/SSC or lineage gating. These approaches, although in their infancy, have the ability to simultaneously detect $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes. There is little data available at present comparing its performance to the above three strategies. Thus, any laboratory intending to use four colour analysis should undertake statistical comparisons with their current method (i.e. lineage gating or CD45/SSC) before its introduction, to demonstrate that there are no significant differences. Furthermore, it can only be used with flow cytometers suitably equipped with either an extra laser and/or electronic hardware/software capable of detecting and analysing the fourth fluorochrome.

Recommended gating strategy

The choice of the above gating procedures will ultimately determine the panel used for $CD4^+$ T lymphocyte determination. The panels recommended within these guidelines facilitate the use of internal quality control checks (with the exception of T-gating). Any abbreviated panel, therefore, will result in the loss of these checks. It is important that careful review of the results is undertaken to ensure consistency with previously obtained results. The use of replicate CD3 evaluations can be used to indicate variability in lysis (there should be less than 3% variation between CD3 percentages when using a panel which contains four replicates of CD3). *The recommended gating strategy, for most laboratories, is either CD45/SSC (using three or more separate fluorochromes) or lineage gating.*

Sample preparation

The whole blood lymphocyte staining method is well described. Briefly, peripheral blood is collected in the manner stated earlier and a volume of blood added to premixed three-colour monoclonal antibodies at the concentrations recommended by the manufacturer. If premixed monoclonal antibodies are not commercially available then the user will have to determine the optimum concentrations to use. Each antibody should be titred against each other and compared with those results obtained when using single staining (Reilly 1996). Following incubation (in the dark) the red cells are lysed (using a proprietary lysing reagent) and washed in accordance with the manufacturer's recommendations. Several commercial systems are now available which employ the 'lyse-no-wash' technique, thereby reducing specimen handling, cell disruption and loss due

to centrifugation and washing. If a wash step is included it is advisable to vortex the cells prior to analysis to break up cell aggregates. Cell specimens should be fixed, as described earlier.

Several control steps should be employed in the analysis of $CD4^+$ lymphocytes, including the use of isotype controls, process controls (both reagent and process) and flow cytometer controls (beads) (the quality control of the flow cytometer will be covered in the next section). The use of isotype controls has several advantages, and their use needs to be evaluated in each individual laboratory. They provide assistance, for example, in determining PMT settings, cursor placement and the identification of positive/negative boundaries where continuous antigen expression is present. Disadvantages are that: (i) the antibody concentration may not have the same characteristics as the test antibodies; (ii) the fluorochrome conjugation may be different and may bind differently to individual cell populations; (iii) they are not required when antigen expression is discontinuous; (iv) isotype controls between manufacturers differ and negative peaks must match the test negative peak and (v) there is an extra cost burden to the analysis. *In view of these considerations multi-colour (three or more fluorochromes) immunophenotyping can be performed in the absence of isotype controls, the cursor placement being set using the unlabelled (negative) cell population.* The cursor placement from the initial analysis tube can be used for all other tubes for a given patient's panel.

The use of a process control is recommended to enable the monitoring of reagent performance, staining, lysis and analysis. *Such controls must be run at least once a week (prior to any of the week's work being processed) and preferably on a daily basis. They must also be run (i) if there has been a change in reagent and/or laboratory personnel, (ii) after instrument service and/or instrument calibration or (iii) in any situation where the validity of the technique may be suspect.* Process controls should be used to test the labelling procedure and if possible to test the lysing step. They should also be stable over time (for minimum of 30 days). Target values should have been assigned to these specimens by the manufacturer. *Results obtained from these reagents must be plotted on a Levy-Jennings type plot, thus providing a visual indication of drift or bias over time. The use of a fresh normal specimen must not be used as a process control due to variability between individuals.* However, such material can be used for instrument set-up and correcting for spectral overlap.

Instrument Quality Control

A stream-in-cuvette flow cytometer must be used to process HIV infected material. *Daily calibration of the instrument must be performed to ensure optimal performance.* This procedure is undertaken using commercial beads in order to: (i) monitor the light scatter and fluorescence peak channel coefficients of variation; (ii) monitor light and fluorescence peak channel drift; (iii) monitor instrument sensitivity and; (iv) facilitate compensation set-up to adjust for spectral overlap. All values should be logged daily, together with instrument settings. All settings should be re-established following a change in bead batch or after an instrument service. Beads, however, only provide guidance for the final flow cytometer set-up and optimization of settings is achieved using a fresh normal specimen.

Sample analysis

The choice of gating strategy will influence the method of analysis. If computer assisted analysis is not used, manual gating strategies must be employed. It is preferable that data are acquired ungated and a minimum of 2500 lymphocyte events are collected. If the data is acquired gated, for example using CD45/SSC, then the gate placement should be checked for each tube, in order to adjust for 'tube-to-tube' variation. Marked differences between tubes usually indicates sample preparation error and the tube (or panel) should be repeated. In addition if a monocyte contamination of $> 5\%$ is observed within the gate, then the tube should be repeated. If this problem re-occurs a new specimen should be requested. To facilitate evaluation of problem samples, a tube containing CD14, CD45 and CD3 may be added to determine the extent of monocyte contamination within the CD45/SSC gate. This approach, however, is rarely required when using CD45/SSC gating. The individual operator should define, in his or her own working environment, the specimen acceptance and rejection criteria when using such software. If in doubt repeat staining or analysis of a repeat specimen should be performed.

Data analysis

The procedure for gating strategies has been detailed earlier and data analysis, therefore, will clearly depend on the strategy chosen. If the lineage gating strategy is employed, computer assisted analysis software is available that defines the analysis region and sets the positive and negative quadrant boundaries for fluorescence staining, based upon the isotype control used, and subsequently calculates the lymphocyte subset values for each of the tests. If T-gating, or CD45/SSC gating strategies are employed, the use of isotype controls can be eliminated by using the unlabelled (negative) populations in each tube to set the positive/negative

quadrant boundaries. Cursor settings are determined by using the fluorescence patterns from CD3, CD4 and CD8. Since cells expressing both CD3 and CD4 stain brightly, the discrimination between negative and positive populations allows easy placement of the quadrant boundaries. Furthermore, the cursor placement from this tube can be used for subsequent tubes. As stated earlier, there is no need to correct for non-lymphocyte contamination when using the three described gating strategies.

Data storage

All primary files, worksheets and copy report forms should be kept for a minimum of 6 months. It is preferable to store data electronically. The flow cytometry data should be stored as list mode files. All electronically stored data should be retained for at least 2 years following the death of the patient. Deletion of the data or destruction of paper records should be in accordance with the Department of Health's document HC(89)20 *Preservation, Retention and Destruction: Responsibilities of Health Authorities under Public Records Act* and locally agreed guidelines.

Data reporting

Counts of $CD4^+$ T lymphocytes should be reported as both percentage and absolute values. The use of T-lineage gating procedures, however, will only allow the reporting of T lymphocyte subsets as a percentage of the total T lymphocytes, thereby giving rise to a higher calculated $CD3^+CD4^+$ and $CD3^+CD8^+$ percentages when compared with lineage gating and CD45/SSC gating approaches. Therefore, if T-gating is employed this should be highlighted to the clinician. Wherever possible, gating strategies should be used that allow the lymphocyte subsets to be reported as a percentage of total lymphocytes (i.e. lineage or CD45/SSC gating). *Analysis techniques for the monitoring of patient $CD4^+$ T lymphocyte percentages must be consistent and not varied from T-lineage to other gating strategies without prior discussion with the clinical users.* Calculation of absolute values of lymphocyte subsets is also subject to technical variation. The most precise method (i.e. giving the lowest CV) is that used by the new generation of flow cytometers (e.g. using either precision fluidics or bead technology), which enable absolute values to be calculated directly, thereby obviating the need for an haematology-analyser-derived total WBC. Therefore, wherever possible, the use of such instruments is preferred. If such instrumentation is not available, then haematology parameters derived as stated earlier should be used in the calculation. The haematology values should be calculated from blood drawn at the same time as that used for immunophenotyping. It is important that adequate haematology quality assurance is in place before using such parameters. Data from the relevant monoclonal antibody combinations should be reported (Table 1) with the corresponding reference limits obtained from a normal population and adjusted for age in young children. The reference limits should be obtained from a minimum of 70 normal individuals. These ranges should be checked on an annual basis, or following any change in instrumentation or methodology (including reagents).

Quality assurance

It is expected that laboratories performing $CD4^+$ T lymphocyte testing are fully conversant with all procedures employed. Such laboratories should meet Clinical Pathology Accredited (CPA) (UK) Ltd standards and be accredited as appropriate. In addition, both internal and external quality assurance should be undertaken. Internal quality assurance has been described earlier. Satisfactory performance should be demonstrable in external proficiency testing schemes such as UK NEQAS Leucocyte Immunophenotyping Schemes. All quality assurance activities should be documented.

Key points

1. All HIV patients should have at least one baseline $CD4^+$ T lymphocyte count determined during the 3 months following diagnosis.
2. $CD4^+$ T lymphocyte counts are measured in HIV individuals to determine the need for prophylactic antiviral (0.5×10^9/l) or anti-*Pneumocystis carinii* (0.2×10^9/l) therapy.
3. Use soundex or hospital reference numbers to ensure confidentiality.
4. The risk of infection must be clearly identified on the sample(s) and laboratory request form(s).
5. Stream-in-air flow cytometers should not be used for $CD4^+$ T lymphocyte determination in HIV-infected individuals.
6. Peripheral blood specimens should be collected in EDTA and fully processed within 18 h. Haematology analysis must be performed within 6 h of collection. Specimens must be stored at between 10°C and 30°C.
7. Cell specimens should be fixed in 2% paraformaldehyde, or proprietary cell fixative, for a minimum of 30 min after staining and prior to analysis.
8. Single colour and U.V. microscopy must not be used for $CD4^+$ T lymphocyte determination.
9. Three or four colour lymphocyte immunophenotyping

Table 3. Advantages and disadvantages of lineage gating approach

Lineage gating advantages	Lineage gating disadvantages
Any debris and non-lymphocyte cells are excluded from analysis	It is best suited to instruments with absolute counting capabilities
A complete profile is obtained from three tubes (including isotype controls)	Advanced software and gating techniques are required
A lymphosum is obtained thus providing an internal QA check	The approach utilises SSC as the primary method of discriminating lymphocytes from granulocytes
It is suitable for both paediatric and adult specimens	The use of a single NK marker may present difficulties in NK identification
The T-lymphocyte subsets are reported as a percentage of total lymphocytes	
It resolves basophils from the lymphocyte population	
The cost may be lower than the conventional larger two-colour panel	
There are fewer tubes to handle and process than with a larger two-colour panel	
It increases the number of evaluable samples compared to the use of FSC/SSC gating strategies	

Table 4. Advantages and disadvantages of CD45/SSC gating

CD45/SSC gating advantages	CD45/SSC gating disadvantages
It can be used with instruments that do not provide absolute counts	Added technical expertise is required for machine set-up, data collection and data analysis
The use of anti-CD45 in each tube allows a consistent tube-to-tube gating check	The third and fourth fluorochromes may not be detected by older flow cytometers
No debris is collected in the analysis gate	Some lymphocytes may have weak expression of CD45 and thus be excluded from the analysis
The inclusion of non-lymphocyte contamination in the gate is minimal	Discrimination of lymphocytes from small monocytes may be difficult
It resolves basophils from lymphocytes	
The use of a high purity lymphocyte gate avoids the need for result correction	
Replicate CD3 determinations are obtained	
$CD3^+CD4^+$ and $CD3^+CD8^+$ populations are obtained from two tubes	
The cost may be lower than the conventional larger two-colour panel	
No isotype controls are required	
There are fewer tubes to handle and process than with the two-colour panel	
It increases the number of evaluable samples compared to the use of FSC/SSC gating strategies	
It is suitable for both paediatric and adult specimens	

is the recommended procedure employing either: (i) T-gating; (ii) lineage gating or; (iii) CD45/SSC gating. Wherever possible the use of lineage gating or CD45/SSC gating is preferred.

10 It is preferable that CD4 and CD8 antibodies are phycoerythrin-conjugated.

11 Isotype controls are not required for T-gating and CD45/SSC gating methods.

12 Process controls to monitor reagent, staining and ideally lysis procedures should be used at least once a week, and preferably daily.

13 Flow cytometer performance should be monitored daily using unstained and fluorescent latex beads.

14 Analysis gates should have $< 5\%$ monocyte contamination.

15 $CD4^+$ T lymphocytes should be reported as both per-

centage and absolute values. If T-gating is used the clinician should be notified that the T-lymphocyte subset percentage values are expressed as a percentage of the total T lymphocytes only.

16 Wherever possible, calculation of absolute $CD4^+$ T lymphocyte numbers should be obtained using a flow cytometer which generates these values independently of haematology derived parameters.

17 Laboratories providing a routine $CD4^+$ T lymphocyte enumeration service should meet Clinical Pathology Accredited (CPA) (UK) Ltd standards and be accredited where appropriate.

18 Participation (together with satisfactory performance) in external proficiency testing schemes should be demonstrable.

19 All primary files, worksheets and copy report forms should be kept for a minimum of 6 months. The data should also be stored electronically and retained for at least 2 years following the death of the patient.

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