response to malignancy, myeloid neoplasia arising secondary to therapy for LCH, and a possible clonal relationship.[7] In fact, LCH, along with other 'L' group disorders, is now in itself considered a myeloid neoplasm arising from constitutive activation of the MAPK pathway, commonly due to BRAF mutations,[8] during myeloid differentiation.[9] Hence neoplastic cells display a gene expression profile of myeloid-derived precursor dendritic cells rather than normal skin Langerhans cells.[9] At least three cases of LCH have been reported in association with CMML.[11] In two of these cases the diagnosis of LCH was made based on S100 and CD1a positivity,[10,12] which could also be consistent with the BIDCT subtype of cutaneous CMML. The third case also reported ultrastructural evidence of Birbeck granules. S100 staining is well known to be non-specific; however, CD1a as well as Langerin are also not exclusively expressed by Langerhans cells, with positive staining recently described in mononuclear phagocyte precursors and subsets of cells within dermal and lymphoid tissues.[9] Differentiation of CD1c (BDCA-1) positive dendritic cells into cells with a Langerhans cell phenotype and function, including containing Birbeck granules, has also been demonstrated.[9] When a possible Langerhans cell infiltrate is encountered outside its usual context, careful clinical correlation and expansion of the immunohistochemical panel may be prudent. Testing for BRAFV600E and MAP2K1 mutations may also be useful in difficult cases.[8] In our case, the advanced age of the patient, the lack of significant epidermotropism, mass formation or eosinophilic infiltrate, the subtle monocytosis in the laboratory record in combination with negative Langerin staining indicated that a diagnosis of LCH was unlikely.

This case presents a rarely encountered immunophenotype of MLC with an unusual CD68 negative and S100/CD1a positive immunophenotype as the first presentation of CMML, and highlights the complexity of immunoprofiles in this context. In these cases, a firm diagnosis cannot be derived from the skin biopsy alone and further investigation with full blood count, peripheral blood smears and bone marrow biopsy is crucial.

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Reference ranges of peripheral blood lymphoid subsets by 10-colour flow cytometry based on 69 healthy adults from Australia

Sir,

Peripheral blood (PB) lymphocyte subset analysis is performed routinely in many haematological[1,2] and non-haematological conditions[3–5] as well as having a fundamental role in the research of the body's immune system to infectious/immune stimuli.[6] Until recently, the analysis was performed using a 4-colour flow cytometer (FCM) using an established panel of fluorochrome-conjugated antibodies. In recent years, single-tube 10-colour FCM has been used to enhance analytical capabilities. There are many benefits to 10-colour FCM, including greater inferential content[7,8] the ability to analyse rare lymphocyte subsets such as CD3+/CD4+/CD8+ T-cells (i.e., double-positive cells, DPC) and CD19+/CD5+ B-cells, and novel subsets within the lymphoid compartment which are outside the classical designations of T, B and NK cells. A single-tube 10-colour approach also has potential to provide a comprehensive subset profile when performing analysis on small volume samples such as paediatric samples.

Local reference values for the normal population are important for reporting quantitative analysis of PB lymphocyte subsets. These reference ranges have been published by international authors, and have mostly used analysis by 4-colour FCM. A recent large, population-based study from Germany[9] reported these reference ranges by using 10-colour FCM. In addition to these publications it is also important to have local data as well as comparison data between 4-colour and 10-colour FCM for smaller or resource-challenged laboratories that may find it difficult to justify the purchase and running costs of a 10-colour FCM instrument. Thus, comparison data between 4-

colour and 10-colour FCM would be useful for these laboratories to report their data with confidence.

We collected data from the PB samples taken from a local Australian healthy blood donor population using single-tube 10-colour FCM. We calculated normal ranges for both common and rare lymphocyte subsets with the aim of providing local data on the normal reference ranges applicable particularly to the Australian population, to see how these data compared to international data, and to compare them to our legacy 4-colour method.

Eighty-eight healthy volunteers were enrolled from both a local Australian Red Cross Blood Service (ARCBS) donor centre and through the Walter and Eliza Hall Institute (WEHI) Melbourne volunteer blood donor registry (VBDR). Appropriate ethics approval and consent was obtained. The two locations were chosen as both centres mandated individual health questionnaires prior to blood donation. After analysis, 19 samples were discarded due to problems with incomplete data sets. This left a total of 69 samples for data collection.

The median age of the population was 45 years with a range of 20−65 years, and comprised 28 (41%) females and 41 (59%) males.

Blood samples were collected into Lithium Heparin tubes (BD Vacutainer; Becton Dickinson, USA). These samples were collected in the morning and processed within 8 hours. Aliquots of whole blood were labelled by direct immunofluorescence in a lyse/no-wash method using established protocols for fluorochrome conjugated monoclonal antibodies. Absolute cell counts were obtained by the addition of fluorescent microbeads of a known concentration (Flow-Count Fluorospheres; Beckman Coulter, USA).

A series of antibody panels were prepared and analysed to yield maximum information about possible methodological variations and also to elucidate rare subpopulations within the lymphoid compartment. In 4-colour experiments, data were acquired on a FC500 flow cytometer (Beckman Coulter) and for 10-colour experiments on a 3-laser/10-colour Navios flow cytometer (Beckman Coulter). In 10-colour experiments, absolute count values were subsequently obtained by incorporating fluorescent microbeads of known concentration (Beckman Coulter Flow-Count beads) and calculating the absolute cell concentration from the ratio of cells:beads using Kaluza software (Beckman Coulter). The design of the project and the operational parameters are summarised in Table 1.

Samples were analysed on a Beckman Coulter FC500 for 4-colour experiments and a Beckman Coulter Navios 10-colour flow cytometer for single-tube 10-colour experiments. Gating was performed using a manual standardised gating protocol as follows: exclusion of air-bubbles (time versus sideward scatter) and exclusion of double events (forward scatter). We used both single-platform and dual-platform analysis (Beckman Coulter LH750) to calculate the total white cell count (WCC) and absolute lymphocyte count. We then gated the lymphocyte percentage (high CD45+ and low side scatter) and calculated the absolute count from the WCC.

Lymphocytes were subdivided into CD3+ and CD3− populations. The CD3− population was further subdivided into CD3−/CD16+ and CD3−/CD56+ NK cells, and CD3−/CD19+ B-lymphocytes. The CD3+ lymphocytes were subdivided into CD3+/CD4+ T-helper lymphocytes, CD3+/CD8+ T-cytotoxic lymphocytes, CD3+/CD5+ T-lymphocytes and CD3+/CD7+ T-lymphocytes. In addition, we sought to gain more information on the frequency of rare subsets that fall outside the usual definitions for T, B and NK cells but which can be found in the lymphoid compartment and may be expanded in some reactive or neoplastic processes. To investigate these subgroups we analysed for CD3+/CD4+/CD8+ T-lymphocytes (or double-positive T-lymphocytes, DPT), CD3+/CD4−/CD8− T-lymphocytes (or double-negative T-lymphocytes, DNT), CD3+/CD16+ T-lymphocytes, CD3+/CD56+ T-lymphocytes, CD3−/CD2−/CD16+ T-lymphocytes, CD3−/CD2−/CD56+ and CD3−/CD19+/CD5+ B-lymphocytes.

We also performed analysis of the standard lymphocyte subsets by 4-colour FCM. We used a similar gating method to that of 10-colour FCM but defined NK cells as CD3−/CD16+/CD56+.

From our analysis we are able to provide reference ranges for the absolute lymphocyte count along with the eight (Table 2) standard subsets and seven rare subsets (Table 3) using 10-colour FCM. There were no differences found among these subgroups based on age and gender.

In addition to analysis by 10-colour FCM, 4-colour FCM analysis of the standard subsets was also performed. Comparison of the results between the two methods was

Table 1 Experimental design and operational parameters

	Legacy 4-colour panels		10-colour panel with CD45 v log SS gating
	3-tube method with CD45 v log SS gating	Tetrachrome method sequential gating[a]	
Events collected in lymphoid gate	5000	6000[a]	5000
Absolute calculation	Lymphocyte count from haematology analyser LH750	Lymphocyte count from haematology analyser LH750	Single platform absolute
Positivity determined	Isotype control	Internal negative controls	Internal negative controls
Antibodies included	CD45 PC7, CD3 PC5, CD8FITC, CD4 PE, CD3 FITC, CD19 PC5, CD16+56 PE	CD45 PC7, CD3PC5, CD4FITC , CD8PE	CD45 Krome Orange, CD16 FITC, CD4 PE, CD5 ECD, CD56 PC5.5, CD2 PC7, CD3 APC, CD7 APC700, CD19 APC750, CD8 Pacific Blue

APC, allophycocyanin; APC700, Allophycocyanin-AlexaFluro700; APC 750, Allophycocyanin-AlexaFluor750; ECD, R-phycoerythrin-Texas Red; FITC, fluorescein isothiocyanate; PC5, R-phycoerythrin-Cyanine5.1; PC5.5, R-phycoerythrin-Cyanine5.5; PC7, R-phycoerythrin-Cyanine7; PE, R-phycoerythrin.
[a] Utilising Tetra CXP automated software.