Electronic Supplementary Information

1.1 Flow cytometry

Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (BD Biosciences, Oxford, UK) with data analysis performed using FlowJo software (Tree Star Inc, Ashland, USA). At least 20,000 discrete events were collected per sample and the percentage positive expression for each mAb was defined using unstained and isotype-matched controls. Based on the light scattering characteristics of each sample population, dead cells, cell debris, and aggregates were eliminated from further phenotypic analysis. For dual-color phenotypic analysis and enumeration, the gating combinations were defined as Stro-1^+/ALP^-, Stro-1^+/ALP^+, and ALP^+/Stro-1^-.

1.2 Flow cytometry analysis

When detecting rare cells within heterogeneous populations, it is crucial that one be able to objectively discriminate between true staining of cells by immunofluorescence methods and experimental staining artefacts that include dead cells, aggregates, non-specific staining, and autofluorescence. This is particularly important for flow cytometry-based methods where accuracy and interpretation of the results are defined in large part by the expertise of the user. Accordingly, numerous steps were taken to improve the accuracy and reproducibility of the analytical data relating to the cell populations under study. First, mouse serum was used to block non-specific Fc receptor binding sites present on the surfaces of cells, thereby reducing the number of false positives present in each analysis. Second, cell debris and staining aggregates were eliminated from further analysis based on their distinct light scattering characteristics **(Figure 5a)**. Third, to reduce high levels of autofluorescence exhibited by periosteal cells, the APD operating voltages were decreased so that >85% of the signals from the unstained cells appeared in the first decade of each fluorescence channel (**Figure 5b**). Attempts to further decrease the voltage resulted in a significant reduction in instrument sensitivity and an inability to discern positive staining. Lastly, isotype controls matched to each primary mAb were used to set the gates used in determining the true proportions of cells positively expressing each antigen (**Figure 5c**). For each antibody or antibody combination, positive expression was defined as the percentage of stained cells with fluorescence levels $\geq$ 99% of the corresponding isotype-matched control antibody stained cells.

Flow cytometric analysis of periosteal cell light scattering characteristics revealed the presence of a heterogeneous mixture of cells with varying sizes (FSC) and granularity (SSC) (**Figure 5a**). This observation confirms previous periosteal cell characterisation studies, which detailed the presence of morphologically and phenotypically heterogeneous cell populations. The immunophenotypic analysis, presented here, revealed three distinct cell phenotypes based on the differential expression of antigens Stro-1 and ALP. A representative example of the gating schemes and resulting expression levels of each target phenotype present in periosteal cells derived from one donor is shown in **Figure 5**. Across all donors, periosteal cells demonstrated a heterogeneous pattern of expression for both the Stro-1 and ALP antigens. The majority (>70%) of periosteal cells demonstrated no detectable levels of Stro-1 or ALP at the surface (Stro-1$^-$/ALP$^-$). A high proportion of the remaining periosteal cell populations expressed the osteoblastic maker ALP exclusively (14.1 ± 11.2%, mean ± SD), while only minor populations were found to express the Stro-1 antigen (3.4 ± 3.6%). While inter-donor variations in phenotype were found, this experimental set-up and gating system enabled us to objectively and reproducibly identify and enumerate distinct cell phenotypes in heterogenous populations.

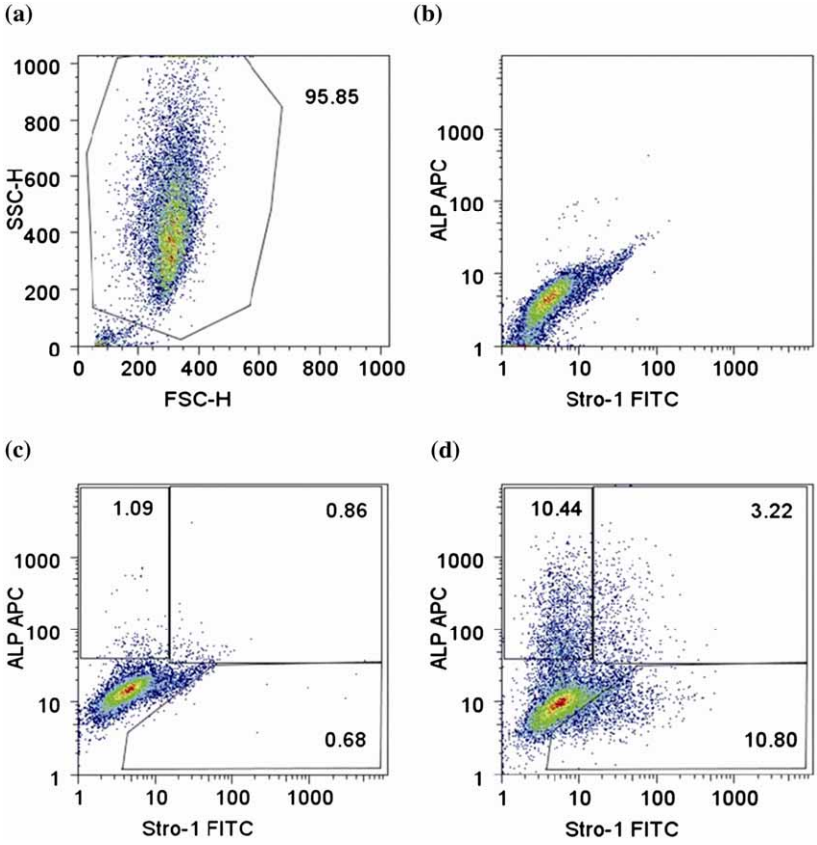

Figure 5 Representative dot-plot histograms demonstrating the light scattering characteristics (a), autofluorecence (b), and immunoreactivity of isotype controls (c) and mAbs ALP and Stro-1 (d) with human periosteal cells derived from a 73 yr old female. Dual color flow cytometric analysis identified the presence of three distinct phenotypes (d), namely Stro-1^{+}/ALP^{-} (lower right gate), ALP^{+}/Stro-1^{-} (upper left gate), and Stro-1^{+}/ALP^{+} (upper right gate). To determine the relative population size of each phenotype, >20,000 events were collected per sample and positive expression was set as the level fluorescence ≥99% of the isotype control (d).