

Title Cytometric gating stringency impacts on studies of type 2 innate lymphoid cells in asthma.

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Type 2 innate lymphoid cells (ILC2) are lineage-negative cells which lack an antigen specific receptor and have been shown in animal models to be a major innate source of type-2 cytokines including interleukin (IL)-5 and IL-13 (1). In several murine models of allergic airways inflammation, using type 2 cytokine-reporter mice and adoptive transfer experiments, ILC2s induced airway eosinophilia (2, 3), expression of IL-5 and -13 (1) and airway hyper-responsiveness (AHR) (4). However, to date, there remains little robust evidence implicating ILC2s in human disease, particularly from patients with asthma (5).

Recently several groups have published reports of increased frequencies of ILC2 cells in human asthma in blood (5-8), sputum (5) and bronchoalveolar lavage (9, 10) defining these

cells as lineage (Lin) negative and positive for CD45, IL7R α (CD127) and either CRTH2 (6-8, 11) or ST2 (5, 10). However, ILC2s constitute a rare population of cells (7, 11) – 0.01-0.03% of total peripheral blood mononuclear cells (PBMCs) (7) – which makes their accurate enumeration in blood and tissues technically challenging (11). Unless assiduous attention is paid to precise setting of Boolean gates during flow cytometry, artefacts will occur, which may have major impacts on the accuracy of data. A particular pitfall in the definitions of ILC2s relates to the use of lineage stains to exclude lineage-positive lymphocytes, monocytes, eosinophils and neutrophils (1). Such stains incorporate a diverse cocktail of multiple monoclonal antibodies conjugated to a single fluorochrome. Due to differences between each marker in cell-surface abundance and antibody affinities and staining intensity, the lineage-positive population, in some cases, could incorporate several discrete regions of high density on a flow cytometry histogram (Figure 1A). Therefore, lineage-negative populations must be gated carefully, referring to the lowest intensity group but not populations with intermediate staining (the populations below the highest intensity). Analysis of published representative flow cytometry plots in several recent reports on human ILC2 cells suggests that discrepancies between recent studies may be related to contamination of “lineage-negative” populations by cells which stain with low antibody intensity, but which are not truly lineage-negative (5-7).

We usually stain human peripheral blood mononuclear cells (PBMC) with typical components of a lineage cocktail (antibodies to CD3(clone OKT3), CD4(OKT4), CD8(RPAT8), CD14(MP ϕ 9), CD16(3G8), CD19(SJ25C1), CD56(H130), CD123(32703), CD11c(BU15), CD11b(DCiS1/18) and Fc ϵ R1(AER-37)). To demonstrate the potential risk of contamination with lineage-positive cells if using an incorrect gating strategy, we stained CD3 independently from a lineage panel with a different fluorochrome (Figure 1A, left panel). PBMC were obtained from healthy volunteers after written consent and approval from Leicestershire, Nottinghamshire Rutland Ethics Committee, UK (08/H0406/189). Figure 1A, middle panel shows that the intensity of CD3 staining using this antibody is located at the same level as the low intensity population of lineage staining. Therefore, although antibody-dependent, if this CD3 antibody was included in the lineage cocktail, T cells would be abundant within the population which stains with low intensity with the lineage cocktail. T cells could therefore potentially be incorporated within the “lineage-negative” gates used in some studies. This potential risk can be confirmed by an additional staining on TCR $\alpha\beta$ (clone

IP26) a marker of T cells, in the antibody panel (Figure 1B). Some $\text{TCR}_{\alpha\beta}^{+}$ T cells (Figure 1B, middle panel) would be included if using an incorrect lineage gating in our routine ILC2 cell staining (Figure 1B, left panel). True ILC2 cells are identified by gating only on $\text{CD45}^{\text{high}}$ cells without significant lineage staining, setting gates by reference to the $\text{CD45}^{\text{high}}\text{Lin}^{-}$ populations (Figure 1A) as, done by Eastman *et al* in their recent publication (12). Consequently, where using some published gating strategies significant populations of CD4^{+} T helper 2 and CD8^{+} cytotoxic T 2 cells (which are also positive to $\text{IL7R}\alpha$ and CRTH2) would likely be included within the ILC2 population. Interestingly no association between ILC2 frequencies and asthma was observed when using a rigorous approach in which PBMC were first depleted of T and B cells using anti-CD3, anti-CD19 and anti-CD14 prior to lineage staining (11).

In summary, given the rarity of these cells in peripheral blood, and the technical challenges of accurately enumerating cells in human airway samples with limited cell numbers, we suggest discrepant studies should be interpreted with caution and careful attention should be paid to the stringency of gating strategies applied in future studies of this intriguing cell type.

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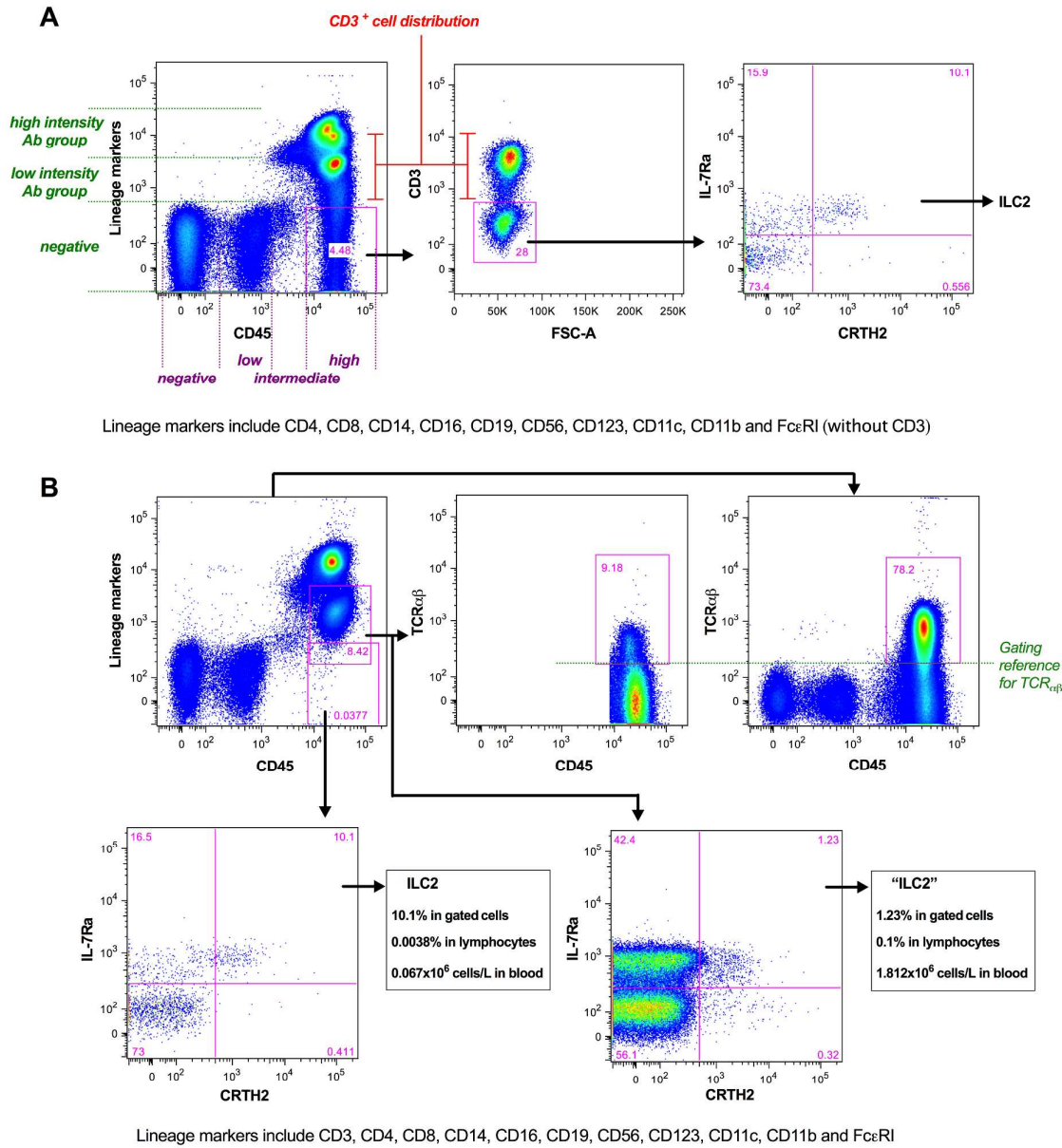


Figure 1. Gating for innate lymphoid cells must exclude low intensity lineage staining. (A) Human PBMCs were stained with CD3 (BV650), CD45 ((H130)PE-Cy7), IL-7Rα (eFluor450), CRTH2 ((BM16)PE) and lineage cocktail (PerCP) excluding CD3. Lineage forms three major distinct populations: high intensity staining, low intensity staining and negative populations (A, left panel). The intensity of the staining on the CD3⁺ population is similar to the low intensity population by lineage cocktail staining (A, middle panel). Gating

of CD3⁻ cells from *A*, middle panel to identify true Lin⁻CD45⁺IL7R α ⁺CRTH2⁺ (ILC2) cells (*A*, right panel). (*B*) Human PBMCs were stained with the same antibody panel as in (*A*) except that CD3 was included in the lineage fluorochrome channel and TCR $\alpha\beta$ (APC) was added. Gating of Lin^{low intensity}CD45⁺ population (*B*, upper left panel) led to contamination by TCR $\alpha\beta$ ⁺ cells (*B*, upper middle panel). TCR $\alpha\beta$ ⁺ cells were gated by reference to (*B*, right panel). Lower panels show relative proportions of ILC2 cells according to the two different gating strategies.