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Authors: Trabanelli S, Gomez-Cadena A, Salomé B, Michaud K, Mavilio D, Landis BN, Jandus P, Jandus C

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Human Innate Lymphoid Cells (ILCs): towards a uniform immune-phenotyping

Running Title: hILC phenotype

Authors: Sara Trabanelli[1*], Alejandra Gomez-Cadena[1*], Bérengère Salomé[1], Katarzyna Michaud[2], Domenico Mavilio[3], Basile Nicolas Landis[4], Peter Jandus[5], Camilla Jandus[1].

[1]Department of Fundamental Oncology, University of Lausanne, Lausanne, Switzerland; [2]University Center of Legal Medicine, University Hospital of Lausanne, Lausanne, Switzerland; [3]Unit of Clinical and Experimental Immunology, Humanitas Clinical and Research Center, Rozzano-Milan, Italy; [4]Rhinology-Olfactology Unit, Otolaryngology Head & Neck Surgery Department, University Hospital and Medical Faculty, Geneva, Switzerland; [5]Division of Immunology and Allergology, Department of Medical Specialities, University Hospital and Medical Faculty, Geneva, Switzerland.

[*]Equal contribution

Dr. Camilla Jandus, MD, PhD

Department of Fundamental Oncology

University of Lausanne

Chemin des Boveresses, 155

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1066 Epalinges, Switzerland

Email: camilla.jandus@chuv.ch

Tel: (0041) 021 692 59 93

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Abstract

Helper Innate Lymphoid Cells (ILCs), the most recently identified population of the Innate Lymphoid Cell family, play a fundamental role in the restoration of tissue integrity, in the protection against infiltrating pathogens as well as in tumor immune-surveillance. ILCs have been divided into three main subsets, ILC1, ILC2 and ILC3, that can be specifically activated by different signals coming either indirectly from pathogens or from other cell populations, including cancer cells. Following activation, ILCs are in turn able to promptly secrete a wide range of soluble mediators that modulate effector cell functions. The discovery and the study of these immune cells is now offering important opportunities for innovative therapies of allergic airway diseases, inflammatory disorders and might be crucial for the discovery of new targets for the therapy of cancer. It is therefore fundamental that the scientific community establishes harmonized guidelines to obtain a consensus in the identification and phenotypical and functional characterization of ILCs.

Introduction

Beside natural killer (NK) cells and lymphoid tissue inducer (LTi) cells, discovered respectively in 1975 and 1997(1,2), several distinct Innate Lymphoid Cell (ILCs) populations have been recently identified(3). As NK and LTi, ILCs require the common chain of the interleukin-2 (IL-2) receptor and the transcriptional repressor Id2 for their development, and are characterized by the absence of rearranged antigen-specific receptors(4-6). These ILC populations have been named ILC1, ILC2 and ILC3 according to their cytokine and transcriptional profiles that mirror the ones of $CD4^+$ T helper (Th)1, Th2 and Th17/22 cells, respectively, and are therefore considered "helper" ILCs(7,8). According to this classification, LTi have been included in the ILC3 population, while NK cells are part of the ILC1 subset, even if they represent the "cytotoxic" ILCs, being the counterpart of $CD8^+$ cytotoxic T cells(9) (**Fig.1**).

Helper ILCs are circulating in the peripheral blood but are also present in lymphoid organs and are enriched at mucosal and barrier surfaces. As innate immune cells, their role is crucial during the early stages of the immune responses(10-12), when they constitute the main source of several pro- and anti-inflammatory cytokines (**Fig.1**). Since ILCs are now the focus of extensive investigations describing their central role in both homeostatic and pathophysiological processes, a careful identification of their phenotype as well as of their function will be key for their understanding.

Identification of ILCs

The first concern in the study of ILCs is represented by the fact that several groups have used distinct criteria and markers for their identification. Indeed, ILCs have been initially defined as Lineage negative (Lin^-) $CD127^+$ cells, but some groups have recently identified non-classical human ILCs $CD127^{dim}$ and mouse ILCs $CD127^-$ (that are out of the focus of this review)(13-16).

Moreover, it is difficult to determine what "Lineage" means for the different research teams. In humans, the most common Lineage markers include CD3, CD19, CD56, CD14, HLA-DR and CD34, to respectively identify T, B and NK cells, monocytes, dendritic cells and hematopoietic progenitor cells. However, these markers might not be sufficient to exclude Lineage positive cells from the ILC gate. For example, it is known that CD3 can be internalized and, therefore, its extracellular expression consequently be reduced once T cells get activated(17), opening the possibility that $CD127^+$ T cells could be included in the $CD3^-$ gate and, thus, making CD3 insufficient for T cell exclusion. Moreover, CD19 might also not be sufficient to completely exclude all human B cell subsets(18), while CD56 and HLA-DR should not be included in the Lineage being expressed on a subset of ILC3(19).

Since there is not a way to precisely determine which markers belong to the "Lineage", different groups have developed their own "Lineage mix". As a consequence, results obtained from different groups are not comparable and/or reproducible. To test the impact of using different Lineage mix on the reproducibility of the results, we collected blood from three healthy donors and we compared the frequency of total ILCs obtained with our Lineage mix and with the mix published by four other groups. As shown in **Figure 2**, by using different Lineage mix, the frequencies of gated ILCs are indeed different, irrespective of inclusion or exclusion of the $CD127^{dim}$ ILCs.

The choice of the Lineage mix

To determine which markers need to be included in an optimal Lineage mix we started with a basic cocktail comprising CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20 and CD34 and we tested which cells were included in the Lin^-CD127^+ region, that were not *bona fide* ILCs.

Since dendritic cells are identified as $Lin^-HLA\text{-}DR^+$ cells, but HLA-DR can also be expressed by ILCs(19), we assessed the expression of other DC markers in the Lin^-CD127^+ population. The two main populations of myeloid DCs are $CD11c^+CD16^+$ or $CD11c^+CD1c^+$, while plasmacytoid DCs are $CD11c^-CD123^+$[20]. Since CD16 was included in our mini-cocktail, we tested only CD1c and CD123. As shown in **Figure 3A**, by comparing Lin^-CD127^- and Lin^-CD127^+ cells, we did not observe a population of $CD1c^+$ or $CD123^+$ in the ILC gate. Notably, for dendritic cell exclusion, beside $CD1c^+$ or $CD123^+$, we decided not to include CD11c in the Lineage since ILCs are developmentally related to NK cells and it has been shown that NK cells can express this marker(21).

Secondly, we wanted to address if basophils or mast cells were contaminating the ILC gate. To test this hypothesis, we monitored the expression of Fc **RI and CD203c** in the Lin^-CD127^- and in the Lin^-CD127^+ gates(22). As shown in **Figure 3A**, we found both a Fc $\mathbf{RI}^+$ and a $CD203c^+$ population in the ILC gate, and therefore we considered necessary to include these two markers in our Lineage mix.

Finally, we tested if myeloid derived suppressor cells (MDSCs) were present in the gate of ILCs. Since we had already included CD14 and CD15 in the Lineage cocktail, we only verified the expression of CD33 in the Lin^-CD127^+ and in the Lin^-CD127^- regions(23). As shown in **Figure 3A**, we found a $CD33^+$ population in the ILC gate and we thus included CD33 in our Lineage cocktail. Using this Lineage mix, the presence of ILCs within the Lin^-CD127^- population is negligible, as assessed by evaluating the expression of T-bet, GATA3 and RORγt in Lin^-CD127^- cells (**Figure 3B**).

In conclusion, by performing all these necessary, but possibly not exhaustive comparisons, we determined that a Lineage mix useful to exclude rare populations from the ILC gate should

contain at least antibodies against CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD203c and Fc RI.

CD161 and NKp44: should we use them?

Another concern in the identification of human ILCs is represented by the use of the marker CD161. Indeed, some groups showed that all ILCs are $CD161^{+}$ and use this marker in their gating strategy(24,25), while others, including our group, do not add CD161 in the gating strategy(26-28). Since it was shown that CD161 is not present in all ILC subsets, being at least ILC1 and LTi $CD161^{+/-(7,29)}$, we propose not to include this marker in the gating strategy, thus avoiding to exclude from the analysis ILCs that are $CD161^{-}$ (**Fig.4**).

Concerning the phenotype of the three different human ILC subsets, namely ILC1, ILC2 and ILC3, it is well established that ILC1 are $CRTH2^{-}ckit^{-}CD56^{-}$, ILC2 are $CRTH2^{+}ckit^{+/-}CD56^{-}$, while ILC3 are $CRTH2^{-}ckit^{+}CD56^{+/-(7,19)}$. Here, the main concern is represented by the choice of the natural cytotoxicity receptor (NCR) used to discriminate between the two main subsets of ILC3, i.e. ILC3 NCR^{+} and ILC3 NCR^{-}. In humans, the NCR family is made of three molecules: NKp30, NKp44 and NKp46. However, only NKp46 is present in both humans and mice and is expressed regardless of the activation status of the cells(30). Therefore, even if several groups proposed to use NKp44 to define ILC3 NCR^{+} cells(26,31), we and others suggested to use NKp46(27,28,30). Once again, the frequencies of ILC3 NCR^{+} and ILC3 NCR^{-} change according to the choice of the NCR marker used for their identification (**Fig.4**).

The ILC gating strategy and the cytokine production

The main function of ILCs is to rapidly respond to different signals by secreting a wide range of soluble mediators. In particular, ILC1 are activated by IL-12/IL-15/IL-18 to secrete type 1

cytokines (i.e. IFN- , TNF-), ILC2 by IL-25/IL-33/TSLP/PGD2 to secrete type 2 cytokines (i.e. IL-4, IL-5, IL-13) and ILC3 by IL-23/IL-1 to secrete type 3 cytokines (i.e. IL-17, IL-22, GM-CSF, LT-α)(32). As a consequence, ILC1 are involved in type 1 immunity to viruses and intracellular bacteria(16,33), ILC2 in type 2 immunity to helminths and allergens(34,35), while ILC3 in type 3 immunity to extracellular microbes(36,37). Therefore, it is fundamental that the three different ILC subsets identified by the selected gating strategy are sensitive to the subset-specific stimulation and are able to produce the pattern of soluble mediators specific of each ILC subset (**Fig.5**). It has to be noticed that ILCs can also be stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin to induce cytokine production/secretion. However, this stimulation is not subset-specific and therefore the different ILC subsets might display production of soluble mediators that is not occurring under physiological conditions (e.g. ILC2 able to produce IFN-).

The ILC gating strategy in disease

Finally, the selected gating strategy should allow to evaluate phenotypic and functional alterations in ILCs in different disease settings. In that regard, it has been shown that ILC2 play a crucial role in allergic airway disease both in mice and in humans(38,39). In this setting, among other stimuli, ILC2 are enhanced and activated by eicosanoids, suggesting that ILC2 function could be modulated by interacting with metabolites of the eicosanoid pathway(40-42). Given the fact that ILC2 are identified as $CRTH2^+$ ILCs, but that CRTH2 is also expressed by basophils and mast cells(22), to appreciate the increase of ILC2 in allergic patients, it is important that the Lineage cocktail includes markers allowing the exclusion of these cells by using other markers than CRTH2 (e.g. Fc RI and CD203c) (**Fig.6**).

It has been shown that ILCs also play a central role in cancer immunity(30,43,44). For example, in the context of leukemia, ILC1 are increased, although hypo-functional, being less able to produce type 1 cytokines in comparison to their healthy counterpart. In particular, ILC1 were found increased in patients suffering from either acute myeloid leukemia or chronic lymphocytic leukemia(27,45,46). Again, the choice of the Lineage cocktail and of the markers useful to identify ILCs should enable to appreciate the ILC1 increase in leukemic patients, without risking a contamination of the ILC1 subset by leukemic cells or other cell subsets (**Fig.6**).

Concluding remarks

In the last few years, great efforts have been done to characterize the newly discovered family of helper ILCs in tissue and in peripheral blood, in both mice and in humans. However, data generated by different groups are often not comparable due to the choice of the markers used to identify both total ILCs and the different ILC subsets. In this review, we describe our gating strategy to identify human circulating ILCs, providing evidence to support our choice of markers (**Table 1**). However, we believe that a consortium of experts in the field should meet to reach a consensus about which markers have to be included in the Lineage and which ones have to be used to discriminate the different ILC subsets, both for mouse and human ILCs. This would be extremely important not only for fundamental research on ILCs, but also for the rapid application of these findings to translational research. Indeed, the next challenge in the ILC research field is represented by the identification of strategies to target ILCs in human diseases. Therefore, the increasing importance of ILCs in innate responses really deserves that a uniform phenotype is established.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

Figure legends

Figure 1. Innate lymphoid cells and their counterparts in the adaptive immune response. Both the innate and the adaptive lymphocytes arise from the Common Lymphoid Progenitor (CLP). Helper ILC1, ILC2 and ILC3 and cytotoxic NK cells originate, upon cytokine stimulation, from the Early Innate Lymphoid Cell Progenitor (EILP). Helper Th1, Th2 and Th17 and the cytotoxic $CD8^+$ T cells originate from the $CD4^+CD8^+$ Double Positive Progenitor (DP), upon T-Cell Receptor (TCR) and cytokine activation. The signature cytokines for each subset are indicated.

Figure 2. ILC frequencies in peripheral blood and tissues. A) Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll density gradient from 3 healthy donors. Five different Lineage mixes to identify ILCs by multicolour flow cytometry were compared: i) Lineage ILC Mix: CD3 (UCHT1), CD4 (13B8.2), CD14 (RMO52), CD16 (3G8), CD19 (HD237) from Beckman Coulter, CD8 (LT8), CD15 (MEM-158) from AbD Serotech, CD20 (2H7), CD33 (HIM3-4), CD34 (561), CD203c (E-NPP3) and FcεRIα (AER-37) from Biolegend; ii) Mix 1(47): CD3, CD14, CD19, CD34; iii) Mix 2(48): CD3, CD14, CD19, CD20, CD56, CD11c (3.9) and CD123 (6H6) from Biolegend; iiii) Mix 3(49): CD1a (BD, HI149) , CD3, CD11c, CD14, CD16, CD19, CD34, TCRαβ (IP26A), TCRγδ (B1) and BDCA2 (V24-785) from BD, FcεR1, CD123 and iiiii) Mix 4(31): CD1a, CD3, CD11c, CD14, CD16, CD19, CD34, TCRαβ, TCR γδ, BDCA2, FcεR1, CD94 (Biolegend, DX22), CD123.

ILCs were identified within the peripheral blood lymphocyte region on the basis of their forward (FSC) and side scatter (SSC) profiles (FSC low and SSC low) and by excluding from the analysis doublets (FSC H/FSC W dot plot, followed by SSC A/SSC W dot plot) and dead cells (positive for ViViD LIVE/DEAD fixable dead cell stain kit (LifeTechnologies)). Left part:

representative comparison of ILC gating on the same donor according to the different Lineage mixes used. Total ILCs were gated in red as $Lin^{-}CD127^{+}$ cells, while $Lin^{-}CD127^{bright}$ ILCs were gated in blue. Right part: histograms represent the mean ±SD of ILC frequencies using the different Lineage mixes. ILC frequencies can significantly differ depending on the Lineage mix used. **B)** Following CD45-Krome Orange (J.33, from Beckman Coulter) gating, the Lineage ILC mix was also used to identify total ILCs and ILC subsets in tonsils, spleen, axillary, mesenteric and inguinal lymph-nodes, thymus, liver, small intestine and colon from tissue cell suspension from freshly deceased, healthy cadaveric donors. Representative plots are shown. The different ILC subsets were determined according to the expression of CRTH2 vs cKit: ILC1 $CRTH2^{-}cKit^{-}$, ILC2 $CRTH2^{+}cKit^{+/-}$ and ILC3 $CRTH2^{-}cKit^{+}$. Antibodies used: Lineage ILC Mix-FITC, CD127-PerCP-Cy5.5 or BV421(A019D5), cKit-PE or APC (104D2) from Biolegend, CRTH2-BV421 or PE-CF594 (BD, BM16).

The dot plots shown in this review are the result of a minimum of 10^6 MNCs acquired on a Gallios

flow cytometer (Beckman Coulter). Data were analyzed using FlowJoTM software (TreeStar).

Figure 3. The choice of the Lineage mix. A) A minimal Lineage mix (Lin) was used comprising PE-conjugated CD3 (UCHT1), CD4 (13B8.2), CD8 (SFCI21Thy2D3), CD19 (89B) from Beckman Coulter, CD14 (M P9), CD15 (HI98), CD34 (8G12) from BD and CD16 (3G8) from Biolegend. CD127 was used in BV421. Comparison of different marker expression between $Lin^{-}CD127^{-}$ lymphocytes in gray (non ILC population) and $Lin^{-}CD127^{+}$ lymphocytes in red (Total ILCs) for DC (CD1c and CD123), basophils, mast cells and MDSC (Fc RI, CD203c and CD33) exclusion. Antibodies used: CD123-PE-Cy7, CD1c-APC-Cy7 (Biolegend, L161), FcεRI-FITC, CD203c-FITC, CD33-FITC, CD34-FITC, cKit-APC, CD127-BV421, CRTH2-

PerCP-Cy5.5. **B)** Representative histograms comparing T-bet, GATA-3 and RORγt expression between Lin^-CD127^- lymphocytes in gray (non ILC population) and Lin^-CD127^+ lymphocytes in red (Total ILCs). The stainings were repeated in 3 different healthy donors with comparable results. Antibodies used are: Lin-FITC, CD127-PerCP-Cy5.5, RORγt-PE (Q21-559), GATA3-PE-Cy7 (L50-822), T-bet-PE-CF594 (04-46) from BD.

Figure 4. CD161 and NKp44. Representative histograms of CD161 expression in total ILCs (upper part), and of NKp46 and NKp44 expression in ILC3 (lower part) gated using either the Lineage ILC Mix or the Mix 4 (purple and grey histogram respectively). The stainings were repeated in 3 different healthy donors with comparable results. MFI values are shown. Antibodies used: Lin-FITC, CD127-BV421, cKit-PE, CRTH2-PE-CF594, NKp46-PerCP-Cy5.5 (9E2), NKp44-APC (p44-8) and CD161-AmCyan (HP-3G10) from Biolegend.

Figure 5. Specific transcription factors and cytokine production in the different ILC subsets. A) Total ILCs and ILC subsets were stained as in Figure 1 using the Lineage ILC mix, then the cells were fixed and permeabilized (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set) and stained for T-bet, GATA3 and RORγt expression. **B)** Total PBMCs ($3x10^6$ cells/condition) were cultured with different cytokine cocktails overnight in the presence of brefeldin A (2 μg/ml). As negative control, PBMCs were cultured in absence of stimulating cytokines for the same period of time. IL-12, IL-15, IL-18 were used to stimulate ILC1s, IL-25, IL-33, TSLP, PGD2 to stimulate ILC2s and IL-23, IL-1β for ILC3s. All cytokines were use at 50 ng/ml. PGD2 was used at 100 nM. Intracellular expression of IFN-γ, IL-13, and LT-α was evaluated in the 3 subsets. Antibodies used: Lin-FITC, CD127-PerCP-Cy5.5 or PE-CF594, cKit-

APC/Fire 750 (Biolegend, 104D2), CRTH2-BV421 or PerCP-Cy5.5, RORγt-PE, GATA3-PE-Cy7, T-bet-PE-CF594, IFN-γ-PE-Cy7 (4S.B3) and IL-13-APC (JES10-5A2), from BD, LT-α-PE (Biolegend, 359-81-11).

Figure 6. ILC gating strategy in HD and patients. Comparison of blood-derived total ILCs and ILC subset distribution using the Lineage ILC mix in **A)** HD vs allergic patients and **B)** HD vs acute myeloid leukemia (AML) patients (n=3 in all the conditions). Antibodies used: Lin-FITC, CD127-BV421, cKit-APC, CRTH2-PE-CF594.

Table 1. Comparison of marker expression in human ILC subsets.

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	ILC1	ILC2	ILC3 NCR^{+}	ILC3 NCR^{-}	
CD3	-	-	-	-	Lineage
CD4	-	-	-	-	
CD8	-	-	-	-	
CD14	-	-	-	-	
CD15	-	-	-	-	
CD16	-	-	-	-	
CD19	-	-	-	-	
CD20	-	-	-	-	
CD33	-	-	-	-	
CD34	-	-	-	-	
CD203c	-	-	-	-	
FcεRI	-	-	-	-	
CD127	+	+	+	+	
CRTH2 (CD294)	-	+	-	-	
cKit (CD117)	-	+/-	+	+	
CD56	-	-	+/-	+/-	
NKp46	-	-	+	-	
NKp44	-	-	+/-	-	
NKp30	-	-	+/-	+/-	
CD161	+/-	+	+	+	
CD11c	-	-	+/-	+/-	
PD-1 (CD279)	-	-	-	-	
CTLA-4 (CD152)	-	-	-	-	
TIM-3	-	-	+/-	-	

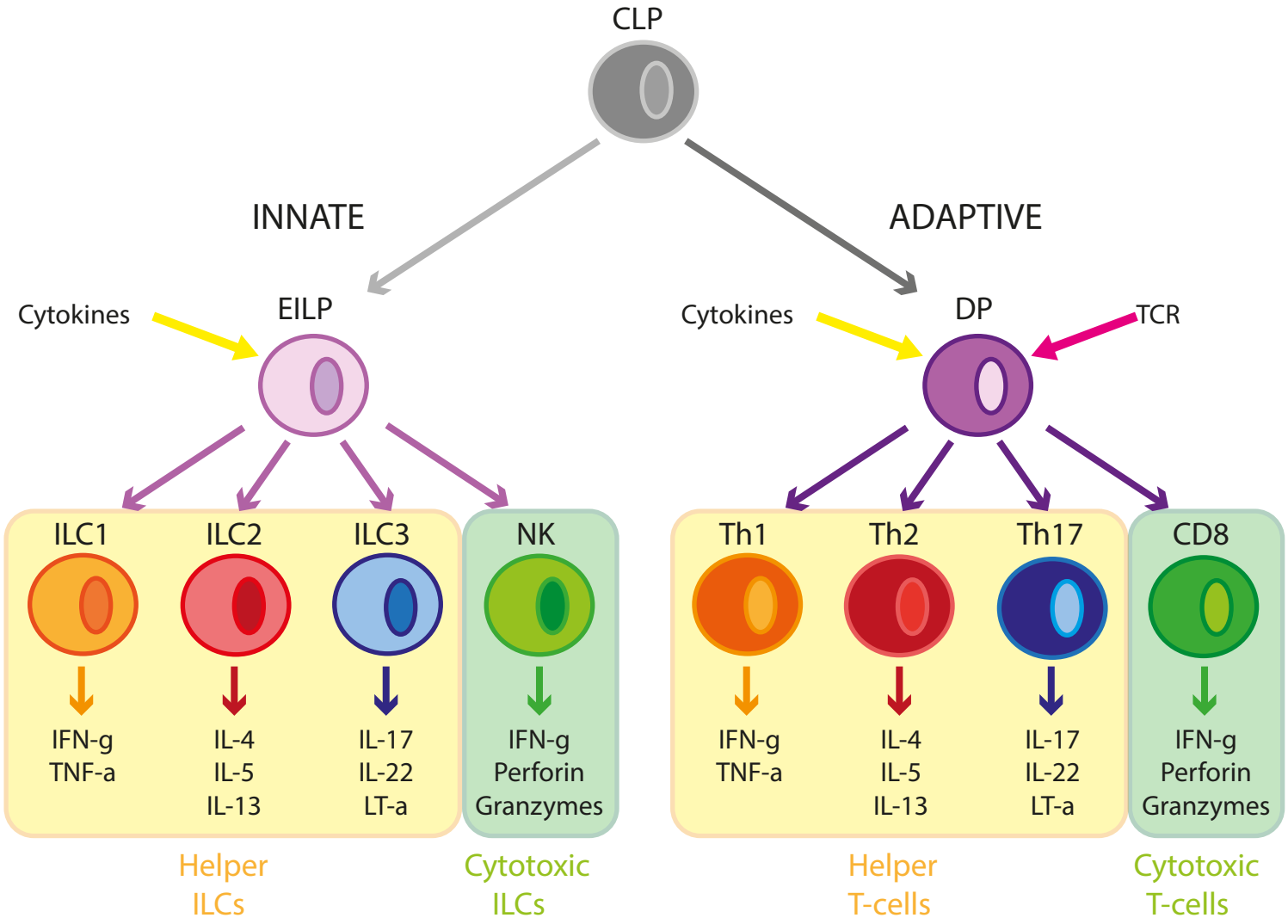

Figure 2

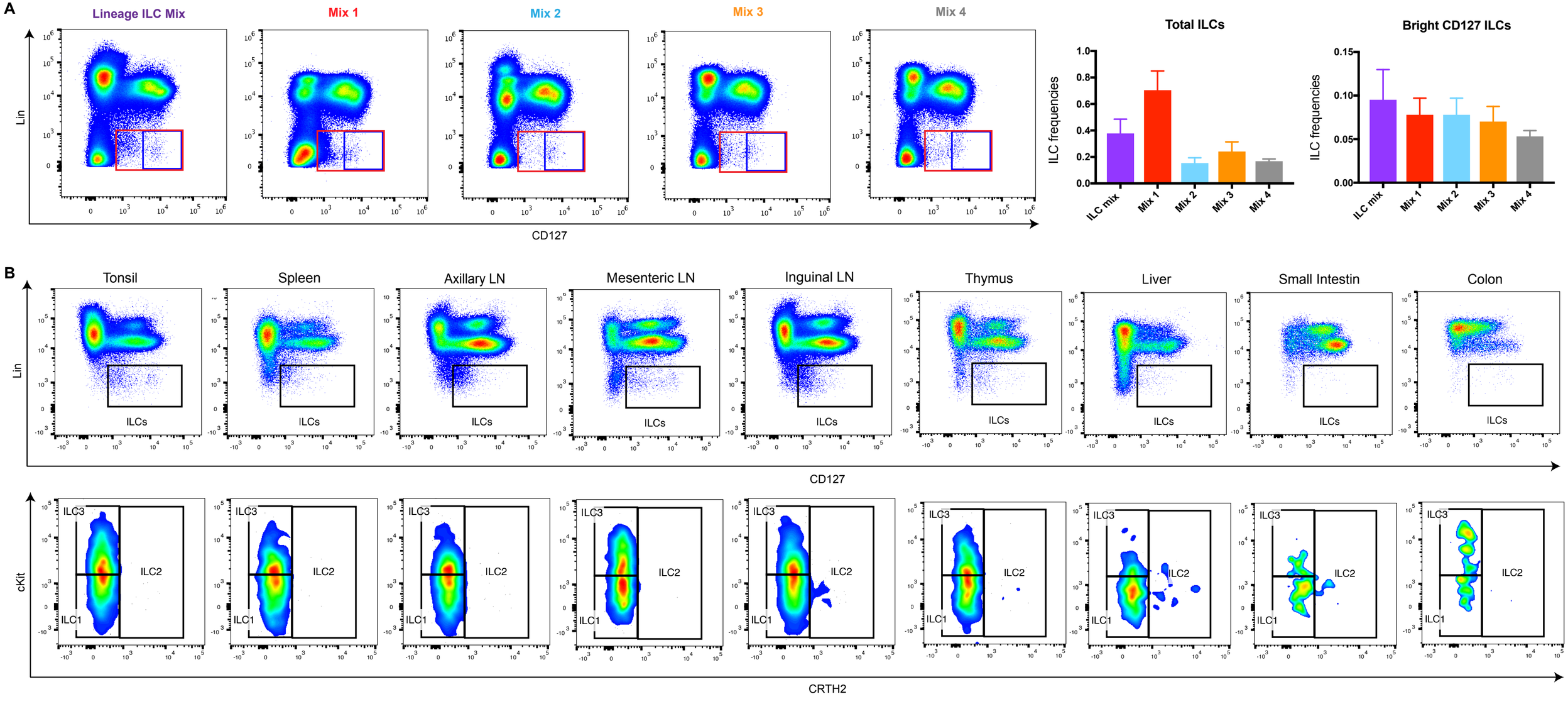

Figure 3

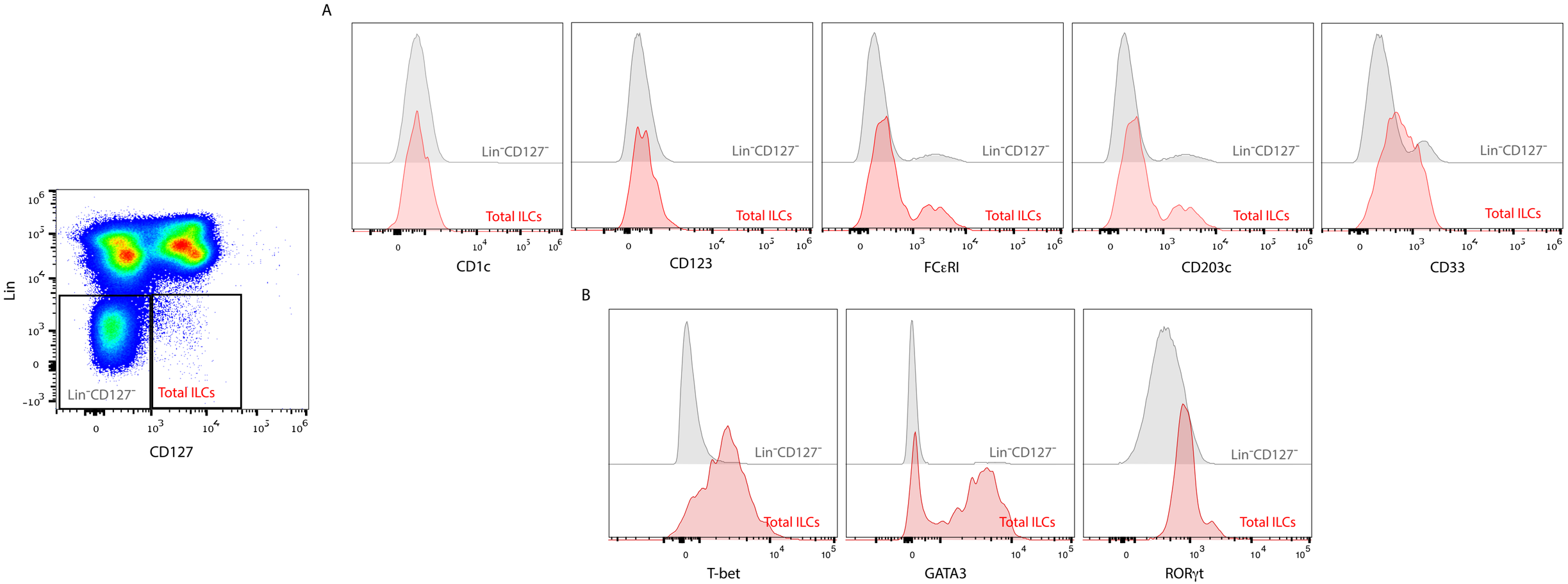

Figure 4

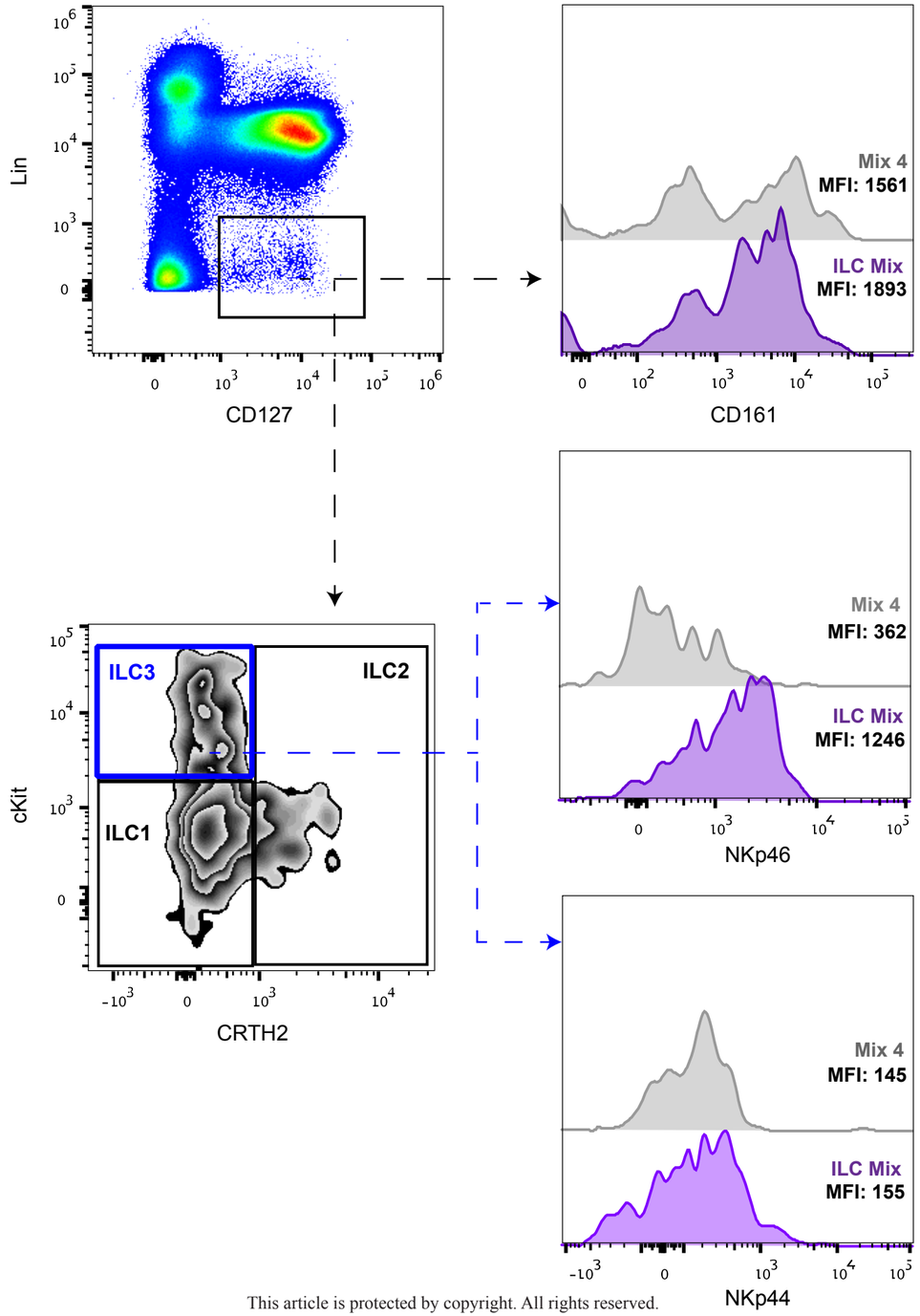

Figure 5

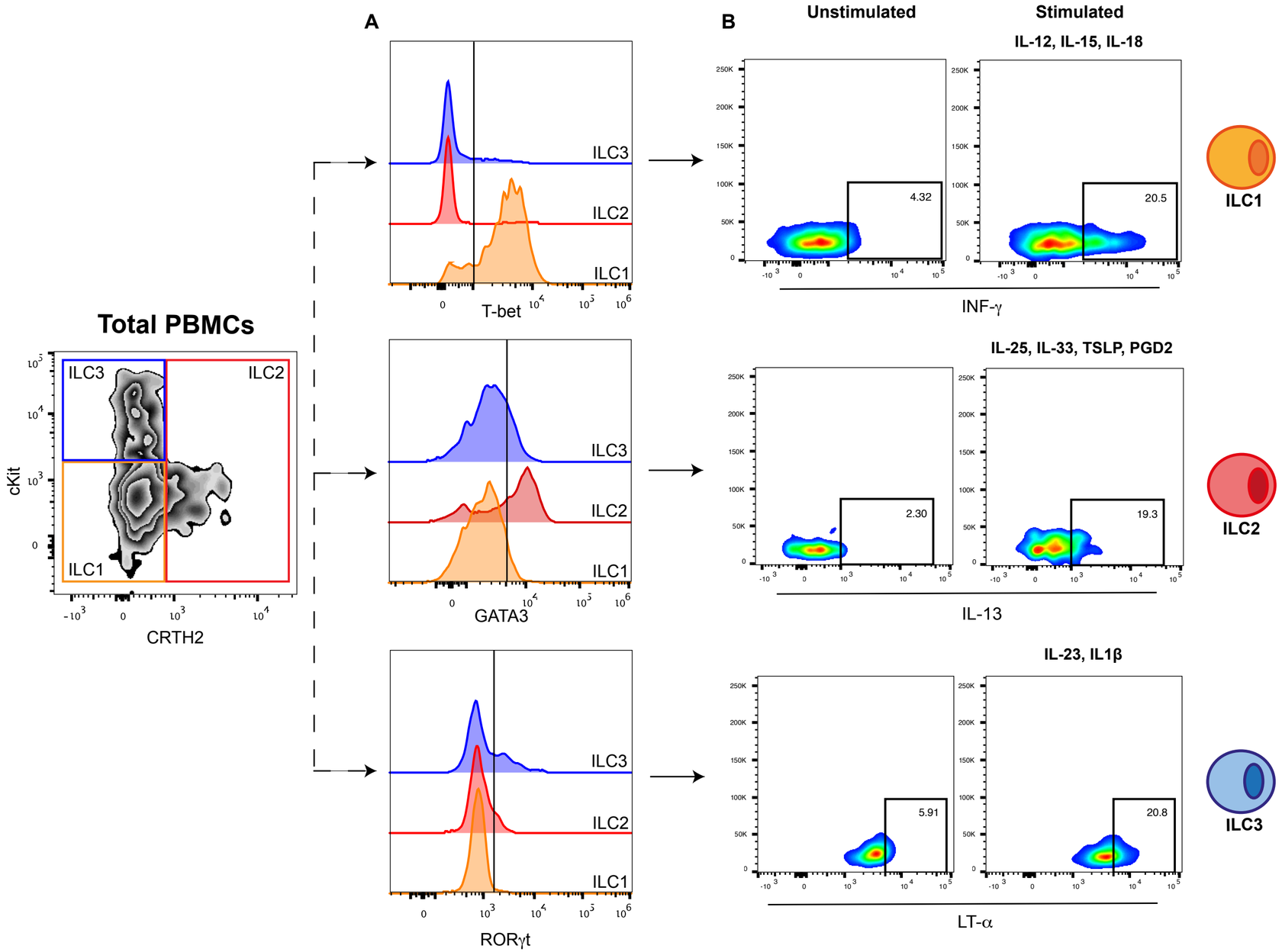

Figure 6