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T Cell Contamination in Flow Cytometry Gating Approaches for Analysis of Innate Lymphoid Cells

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Abstract

Innate lymphoid cells (ILCs) differ from T and B cells as they do not express genetically rearranged antigen receptors. The most prominent member of this group, NK cells, can be identified by numerous surface receptors such as natural cytotoxicity receptors (NCRs). However, novel groups of ILCs have recently been described and classified based on fate-determining transcription factors and cytokines being produced, similarly to T helper cells. Due to the lack of exclusive markers, ILCs are primarily defined by the paucity of lineage markers. Using RORγt-fate-mapping mice, we found that the common lineage exclusion using CD3 yields an ILC population containing a large proportion of T cells with recombined TCR loci and low expression of CD3. Thus, we suggest adding CD5 as a marker for thorough elimination of T cells to avoid erroneous interpretations of ILC function in immunity.

Introduction

The term innate lymphoid cells (ILCs) unifies a group of cells that are developmentally related and lack most lineage markers. They share morphological features with lymphocytes but, in contrast to B and T cells, they do not express recombined antigen receptors and can therefore be classified as innate immune cells [1]. In the past years, ILCs have been shown to be critical for physiological processes like lymphoid organ development [2], tissue homeostasis [3], early control of pathogens [4], but they can also promote inflammation if inadequately activated [5,6]. Based on their cytokine and transcription factor expression profile ILCs have recently been given a uniform nomenclature, dividing ILCs in three main groups and thereby broadly reflecting the classification of T helper cell subsets [7]. The first subset (Group 1 ILCs) depends on expression of T-bet, leading to expression of IFN-γ [8,9]. The second subset (Group 2 ILCs) expresses Gata3 and is characterized by the secretion of type 2 cytokines such as IL-13 and IL-5 [10,11]. Group 3 ILCs (hereafter referred to as ILC3s) depend on the expression of the transcription factor retinoic acid-related orphan nuclear receptor Retinoid acid receptor-related orphan receptor (ROR)γt for their development as well as their function and secrete IL-22 and IL-17 [12,13].

The most abundant ILC subtype are natural killer (NK) cells that can be characterized by expression of specific phenotypic markers, many of them belonging to the family of natural cytotoxicity receptors. However, for more recently discovered ILC subtypes such exclusive markers have yet to be defined. Therefore, during flow cytometric analysis of ILCs usually a combination of lineage markers is used to exclude other immune cells. As the phenotypic appearance of ILCs and their cytokine secretion pattern in many models resemble that of T cells, the elimination of T lymphocytes is absolutely critical to analyze phenotypic and functional properties of ILCs. Hence, many studies have been conducted using animals deficient for the recombination activating genes (RAG) 1 or 2, thereby genetically eliminating B and T cells [5,8].

However, for some experimental models and studies on human tissues this is not an option, thus T cells have to be excluded by their expression of T cell specific markers. Using flow cytometry, separation of ILCs from T cells is most frequently achieved by staining for CD3, a part of the T cell receptor (TCR) complex. As T cells are known to modulate their CD3 expression level in particular following their activation [14] and usually outnumber the rare ILC subgroups, a clear discrimination between these two populations is essential.

Based on exemplary analysis of RORγt dependent ILC3s, we show here that exclusion of T cells using solely gating on CD3 leaves a significant contamination with T cells. These T cells were identified by staining for CD5, a surface marker that is involved in modulating TCR signaling. Depending on the organ analyzed, the fraction of T cells within the gated ILC3 population accounted for up to 80% of the remaining ILC3s, which in certain studies may falsify subsequent results. Quantitative PCR analysis for the constant regions of the T cell receptor α and δ chain (TRA1 and TRDC) revealed that the remaining CD5+ cells belonged mainly to the γδ T cell lineage. In order to obtain a non-ambiguous separation of ILCs and T cells, stainings for different T cell surface molecules need to be combined. We therefore suggest comple-
menting a CD3 and TCRβ staining with an antibody binding the CD5 molecule.

Results and Discussion

CD5+ fraction remains in the ILC3 gate after exclusion of T cells using CD3 and TCRβ

Besides its importance for ILC3s, RORγt is also involved in the development of T cells, mainly T Helper 17 and γδ T cells [13,15]. However, most ILC3 and T cell subsets will subsequently downregulate the expression of RORγt [16]. By breeding a transgenic mouse expressing the Cre recombinase under the control of the RORγ promoter (driving RORγt) to a ROSA26-stop β-gal eYFP reporter mouse yielding in RORγ-eYFP mice, the fate of these YFP-positive RORγt-dependent cells can be mapped [17].

Using leukocytes isolated both from lymphoid (spleen) and non-lymphoid organs (colon and lung), we compared different gating approaches for the efficiency in separating ILC3s and T cells. The gating strategy involved exclusion of doublets, dead cells and B220 expressing events from CD45+ leukocytes. In order to exclude T cells, CD3 and TCRβ positive events were negatively selected followed by gating on YFP+ cells (Figure 1a). Similar gating approaches are used to characterize both murine and human ILCs but are often not depicted in any figure or involve gating out multiple lineage markers in one fluorescent channel [10,18–20]. However, upon further scrutiny of the assumed ILC3s, we discovered a significant percentage of these cells to stain positive for CD5, a molecule described to inhibit the T and B cell receptor signaling and was historically used as a pan T cell marker. Whereas in the colon this frequency was relatively low with 43.9% CD5+ cells within the ILC3 gate, in spleen and lung it reached 78.2% and 68.9%, respectively (Figure 1b). Strikingly, in RORγ-eYFP fate mapping mice deficient for RAG1 no expression of CD5 was detected, suggesting that the CD5+ cells obtained from non-RAG mice belong to the T cell lineage (Figure 1c). Notably, as
CD5⁺ cells remaining within the CD3⁻ TCRβ⁻ ILC3 gate consist of αβ T cells

To determine the nature of the CD5 expressing CD3⁻ TCRβ⁻ cells, this population was purified by flow cytometric cell sorting from the spleen (Figure 1a and b). The mRNA levels for the constant region of the T cell receptor α and δ chain (TRAC and TRDC) were compared to that of CD5⁺ ILC3s, αβ and γδ T cells as well as monocytes. As a genetic control we used ILC3s derived from RORc-eYFP RAG1⁻/⁻ mice. Whereas CD3⁺ TCRβ⁺ CD5⁺ ILC3s showed minor expression of both TRAC and TRDC (probably reflecting inevitable contamination from the sort purification), purified αβ and γδ T cell populations as expected transcribed high levels of TRAC or TRDC, respectively (Figure 2). Strikingly, when analyzing TRAC and TRDC expression levels in CD3⁻ TCRβ⁻ CD5⁺ cells we detected high levels of TRAC, suggesting that this population indeed consisted mainly of αβ T cells.

Conclusions

We conclude that gating on lineage negative cells only using CD3 is not sufficient to firmly exclude αβ T cells from further analysis. This problem becomes particularly evident, when a CD3 containing “dump channel” is employed, where cells expressing lineage markers at different intensities are gated out in a single fluorescence channel. Furthermore, the scarcity of ILCs together with the high number of T cells, which depending on their activation status might express different levels of CD3, seems to cause limits for a separation based on one T cell marker. Thus, results obtained from contaminated ILC populations will be biased and T cells will contribute to the detected cytokine levels. This may not only produce artifacts but also mislead the characterization of ILC subsets according to the nomenclature based on cytokine and transcription factor expression.

Given that within the past years a strong association between ILC and T cell effector function has been proposed, we suggest that a thorough separation between these two populations is essential. Hence, lineage negative gating should not only be performed using CD3 only, but also CD5, preferably in a separate fluorescent channel.

Materials and Methods

Mouse strains

RORc-CRE and ROSA-stop<sup>0.12</sup>-eYFP mice, provided by Andreas Diefenbach, were bred to obtain RORc-eYFP fate mapping animals. Both strains were crossed to RAG1<sup>−/−</sup> mice, purchased from the Jackson Laboratory. Animals were between the ages of 6 to 16 weeks and kept under specific pathogen free conditions. All animal experiments were approved by the Swiss Cantonal Veterinary Office (license 147/2012, Zurich, Switzerland).

Cell isolation

Mice were euthanized using CO₂ inhalation and perfused with 40 ml cold PBS. Spleen, colon and lung were excised and processed as described below. Spleens were homogenized by mechanical disruption and filtered through a 70 μm mesh, followed by lysis of erythrocytes. Lungs were cut into small pieces, followed by 60 min of digestion at 37°C with 1 mg/ml Collagenase D (Roche) and 0.5 mg/ml DNase (Sigma) in IMDM containing 25 mM HEPES and 2% fetal calf serum (FCS). Remaining pieces of tissue were homogenized using syringes and 18 gauge needles, followed by filtration through a 70 μm mesh.
Colons were separated from the mesenteric fat. Luminal mucus was removed mechanically followed by incubation in Ca++/Mg++-lacking HBSS containing 2% of FCS, 1 mM DTT and 1.35 mM EDTA for 15 min at 37°C. After further incubation in HBSS complemented with EDTA for 30 min at 37°C the colons were cut and digested using 0.4 mg/ml collagenase IV (Sigma Aldrich) for 45 min at 37°C. The samples were then homogenized using a syringe with an 18 gauge needle and filtered through a 70 μm cell strainer.

Flow cytometry
All flourochrome-conjugated antibodies used were obtained either from BD, BioLegend or eBioscience, and stainings were performed according to standard procedures for 20 minutes at 4°C. In all stainings, dead cells were excluded using an Aqua Live/Dead fixable staining reagent (Invitrogen), and doublets were excluded by FSC-A vs FSC-H gating. Analysis was performed using a LSR II Fortessa (special order research product, BD) with four laser lines (405 nm, 488 nm, 561 nm and 640 nm). Cell sorting experiments were carried out using a FACS Aria III (BD). Data analysis was done using FlowJo V9.x (Treestar).

mRNA extraction and quantitative real time PCR
Purified cells were resuspended in Trizol (Invitrogen) and kept at -80°C. RNA was extracted using the PureLink RNA Micro Kit (Invitrogen) according to the manufactures. RNA concentration was determined using a spectrophotometer (NanoDrop). Subsequent cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen) and oligo (dt) primers (PeproTech). Quantitative analysis was conducted using a SYBR Green master mix (Roche), TRC and TRDC specific primers (see below) and measured by the C1000 Touch thermal cycler (BioRad); mRNA levels were determined by the cycle threshold values and normalized to the expression of the Pib2 gene. Primers were designed using CLC Workbench: TRC: ACAAGCTTCACCCTGCAAA forward, GCTTTTCCTCAGTGTCACGGG reverse; TRDC: TAGTCTCTCAGTGTCAGGGC reverse, CTAAGACTGGCTGTATTGCAA forward. Statistical significance was determined using the BioRad CFX Manager 3.0.

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Author Contributions
Conceived and designed the experiments: SB KN FM BR. Performed the experiments: SB KN FM. Analyzed the data: SB KN FM. Contributed reagents/materials/analysis tools: BB. Wrote the paper: SB BB. Helped with and performed experiments: SH.

References
1. Spits H, Cupedo T (2011) Innate Lymphoid Cells: Emerging Insights in Development, Lineage Relationships, and Function. Annu Rev Immunol. doi:10.1146/annurev-immunol-020711-070553.
2. van de Pavert SA, Olivier B, Goverse G, Vonderhoff MF, Greuter M, et al. (2009) Chernomikine CXCL13 is essential for lymph node initiation and is induced by retinoic acid and neuronal stimulation. Nat Immunol. 10: 1193-1199. doi:10.1038/ni.1789.
3. Montecelli LA, Sonnenberg GF, Alt MC, Alenghat T, Ziegler CGK, et al. (2011) Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nat Immunol. doi:10.1038/ni.2131.
4. Cella M, Fuchs A, Vemmi W, Facchetti F, Otero K, et al. (2009) A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. Nature 457: 722-725. doi:10.1038/nature07357.
5. Buonocore S, Ahern PP, Uhlig HH, Ivanov II, Littman DR, et al. (2010) Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature 464: 1371-1375. doi:10.1038/nature08949.
6. Chang Y-J, Kim HY, Albacker LA, Baumgarth N, McKenzie ANJ, et al. (2011) The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of Type 2 Innate Lymphoid Cells. Immunity. doi:10.1016/j.immuni.2012.09.008.
7. Scimone G, Hirahara K, Takahashi H, Laurence A, Villarino AV, et al. (2012) Distinct requirements for T-bet in gut innate lymphoid cells. J Exp Med 209: 2331-2338. doi:10.1084/jem.20122097.
8. Hoyler T, Klose CSN, Souabni A, Turqueti-Neves A, Pfleifer D, et al. (2012) The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of Type 2 Innate Lymphoid Cells. Immunity. doi:10.1016/j.immuni.2012.06.020.
9. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, et al. (2012) The Transcription Factor GATA3 Is Essential for the Function of Human Type 2 Innate Lymphoid Cells. Immunity. doi:10.1016/j.immuni.2012.08.015.
10. Satoh-Takayama N, Vosshenrich CAJ, Lejean-Pottier S, Sawa S, Lochner M, et al. (2008) Microbial flora drive interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. Immunity 29: 959-970. doi:10.1016/j.immuni.2008.11.001.
11. Eberl G, Marson S, Sunshine MJ, Rennert PD, Choi Y, et al. (2004) An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. Nat Immunol. 5: 64-73. doi:10.1038/ni1022.
12. Valitutti S, Muller S, Salo M, Lanzavecchia A (1997) Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation. J Exp Med 185: 1859-1864.
13. Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, et al. (2000) Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science 288: 2369-2373.
14. Vonarbourg C, Mortha A, Bui VL, Hernandez PP, Kennedy GD, et al. (2011) Chemokine CXCL13 is essential for lymph node initiation and is induced by Notch. Nat Immunol. doi:10.1038/ni.2187.
15. Lee JS, Cella M, McDonald KG, Garlanda C, Kennedy GD, et al. (2011) AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. Nat Immunol. doi:10.1038/ni.2187.
16. Qi J, Heller JJ, Guo X, Chen Z-ME, Fish K, et al. (2012) The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity 36: 92-104. doi:10.1016/j.immuni.2011.11.011.
17. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, et al. (2012) The Transcription Factor GATA3 Is Essential for the Function of Human Type 2 Innate Lymphoid Cells. Immunity. doi:10.1016/j.immuni.2012.08.015.