

Accepted Manuscript

Type 3 innate lymphoid cells induce proliferation of CD94+ NK cells

Shuo Li, PhD, Hideaki Morita, Beate Rückert, tadech boonpiyathad, Avidan Neumann, Cezmi Akdis



PII: S0091-6749(17)30661-9

DOI: [10.1016/j.jaci.2017.03.033](https://doi.org/10.1016/j.jaci.2017.03.033)

Reference: YMAI 12757

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 4 January 2017

Revised Date: 14 March 2017

Accepted Date: 21 March 2017

Please cite this article as: Li S, Morita H, Rückert B, boonpiyathad t, Neumann A, Akdis C, Type 3 innate lymphoid cells induce proliferation of CD94+ NK cells, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.03.033.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Type 3 innate lymphoid cells induce proliferation of CD94⁺ NK cells

To the Editor:

Recent discoveries on innate lymphoid cells (ILCs) ¹⁻⁶ and innate immune memory ⁷ have brought new insights to elucidate the boundary between innate and adaptive immunity. ILCs are a growing family of immune cells, classified in three different subtypes (ILC1, ILC2 and ILC3) according to their cytokine and transcription factor expression ^{1, 2}. They initiate inflammation by promptly producing pro-inflammatory cytokines in response to multiple stimuli, and can directly influence on a variety of immune cells to orchestrate innate and adaptive immune responses ^{1, 2}. To understand how ILC might bridge innate and adaptive immunity or further contribute to expansion of innate immune cells, we asked whether ILC might affect other immune cells within its surroundings. Using a co-culture model, we discovered that ILC3s negative for natural cytotoxicity receptor NKp44 drive a rapid and dramatic expansion of CD94⁺ natural killer (NK) cells. Our data suggests an important link between these two cells and a novel function of ILC3s, which may have important basic and translational implications.

ILC1s, ILC2s and NKp44⁻ ILC3s were isolated from human peripheral blood mononuclear cell (PBMC) using a protocol previously established in our laboratory. The study was approved by the SIAF ethics Committee and the Zurich University Human Research Ethics Committee. In brief, we gated on lineage⁻ CD127⁺ viable lymphocytes, of which the CD161^{mid/+} cells were the main ILC population. The three ILC subsets were then distinguished based on their differential expression of c-kit, CRTH2 and NKp44 (i.e. ILC1: Lin⁻ CD127⁺ CD161⁺ CRTH2⁻ c-kit⁻, ILC2: Lin⁻ CD127⁺ CD161⁺ CRTH2⁺, NKp44⁻ ILC3: Lin⁻ CD127⁺ CD161⁺ CRTH2⁻ c-kit⁺, NKp44⁻) (Online repository Figure 1 and Online Repository Methods). Although ILC3 has two distinct subsets, NKp44⁻ and NKp44⁺ ⁸, the latter can hardly be detected in peripheral blood in humans, and thus our study using blood samples focused on NKp44⁻ ILC3.

To investigate the interaction of ILC with other immune cells, freshly isolated ILCs were co-cultured with CFSE-labeled autologous PBMC at an ILC/PBMC ratio of 1:20 in U bottom plates to enhance cell-to-cell contact (see Online Repository Methods for details). The cells were cultured for 5 days, a timing consistent with our previous experiences with short-term cultures. We first compared the percentage of dividing cells that showed CFSE dilution within total viable lymphocytes in the presence or absence of ILC. As compared to PBMC alone, we found that some cells in the ILC-PBMC co-cultures were proliferating (showing CFSE dilution, Fig 1, A). The percentage of dividing cells (R1 in Fig 1, A) within total viable lymphocytes gate were significantly higher in cultures with NKp44⁺ ILC3s compared to control without ILC (Fig 1, B). Co-cultures with ILC1 and ILC2 also showed trends of higher proliferation compared to no ILC control (Fig 1, B). The effect of NKp44⁺ ILC3s was particularly notable, where the proportion of dividing cells reached up to 17-32 %, significantly higher than that of ILC1 and ILC2 (Fig 1, B).

Since the ILCs were CFSE unlabeled and would fall in the “dividing” gate, we next addressed whether the dividing cells have originated from PBMC or ILC or both. Phenotyping of the co-culture revealed that the cells were mainly CD3⁺ T cells and CD94⁺ NK cells, but not CD14⁺ monocytes or CD19⁺ B cells (Fig 2, A). We also stained for CD4 and CD8 T cells, as well as CD27, CCR7 and CD16 cells. These data demonstrate that ILC3 particularly induced the proliferation of CD94⁺ NK cells and to some degree the CD3⁺CD4⁺ cells, but slight, if any, proliferation of CD8⁺ T cells, and CCR7⁺ and CD27⁺ cells (Online Repository Fig. 2). CD14⁺ monocytes cannot be reliably detected after 5-day cultures. Their numbers were too small, which likely represent noise.

The frequency of various cell types within the total viable lymphocytes were shown in Fig 2, B, revealing that NKp44⁺ ILC3s had the strongest capacity in promoting CD94⁺ cell expansion. The proportion of dividing CD94⁺ cells within total CD94⁺ parental population was also significantly higher than that of CD3⁺ T cells (Fig 2, C), demonstrating the propensity of CD94⁺ cells to proliferate in response to NKp44⁺ ILC3s. The ability of ILC3 in promoting CD94⁺ cell proliferation seemed to occur in the absence of antigen, as our co-cultures did not contain antigen-specific

1 stimuli. One of the important findings in this study is that neither of the cells were specifically or
2 non-specifically stimulated and the culture conditions represent an autologous and spontaneous
3 interaction. It remains an interesting question, if and how antigen and different innate immune
4 response stimulating substances, such as vaccine adjuvants might affect this process?

5 ILCs do not express CD94, thus the CD94⁺ proliferating cells are NK and to various degree NK
6 – like cells within the PBMC. CD94 is a transmembrane protein belonging to the C-type lectin
7 superfamily. It forms a heterodimer with NKG2 family molecules to transduce cellular signals.
8 CD94/NKG2 is ligand for HLA-E, a non-classical MHC glycoproteins class I that expressed by
9 ILCs (Li et al., unpublished RNAseq data). Different to CD16, which is also expressed by
10 macrophage and mast cells, CD94 is mainly expressed on NK cells. Online Repository Fig. 3
11 depicts CD16 and CD94 expression on freshly isolated PBMC. In human tonsil, “stage III”
12 immature NK cells defined as CD34[−] CD94[−] c-Kit⁺ cells share similar phenotypic markers with
13 ILC3s,⁹ suggesting that these “stage III” immature NK cells might be heterogeneous and
14 potentially composed of ILC3s. It is however unknown whether this knowledge might be applicable
15 to blood ILC3. CD94⁺ cells were the most prominent cell type in response to ILC3-driven
16 expansion, and to a lesser extend CD3⁺ T cells. However, we do not know if ILCs themselves might
17 be proliferating, or other minor cell types such as blood dendritic cells or other cells in the PBMC
18 might be involved in this apparent ILC3 - CD94⁺ cell interaction. We detected a small fraction of
19 proliferating CD94⁺CD3⁺ cells, presumably NK-T cells although their proportions remained minor
20 (data not shown). In some experiments, supernatants of co-cultures were collected and analyzed for
21 cytokine expressions. As shown in Online Repository Fig. 4, ILC3-PBMC co-cultures showed
22 strong trends of increased cytokine secretion compared to ILC1 and ILC2, such as GM-CSF and
23 IFN- γ .

24 The mechanism of the interaction is unknown. Could cell-to-cell contact be required, and if so,
25 what receptor-ligand pairs are involved? Are soluble factors important, and if so, which soluble

factors are necessary and what are their cellular sources? Additional studies are needed to explore the factors driving in NK cell expansion, as well as plasticity between NK cells and ILC3s.

Taken together, our data opens a door to important and interesting new studies. Whereas ILC3s are known to promote innate immunity to extracellular bacteria, our findings also suggest that ILC3s may also be involved in innate immunity against tumors and viral infections by rapid expansion of NK or NK-like cells. NK cell memory was reported in recent studies, but mechanisms remain unknown ⁷. Our findings suggest that ILC3 might play a role in NK cell memory development and/or recall. Such a link will provide opportunities for clinical translations, such as in allergy and cancer treatment.

Shuo Li, Ph.D.^{a,b,*}

Hideaki Morita, M.D., Ph.D.^{a,c,*}

Beate Rückert, Sci Tec^{a,c}

Tadech Boonpiyathad^{a,c}

Avidan Neumann, Ph.D.^{a,c,d}

Cezmi A. Akdis, M.D.^{a,c}

a. Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Obere Strasse 22, CH-7270 Davos, Switzerland

b. Department of Microbiology, Monash University, Melbourne, Victoria, Australia;

c. Christine Kühne-Center for Allergy Research and Education, Davos, Switzerland

d. Institute of Environmental Medicine, UNIKA-T, Helmholtz Center, Munich, Augsburg, Germany

* These authors contributed equally to this work.

Corresponding author: Cezmi A. Akdis, Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos Platz

E-mail: akdisac@siaf.uzh.ch

Tel.: +41 (0) 81 410 08 48

Fax: + 41 (0) 81 410 08 40

Swiss National Science Foundation Grant Number 310030_156823

We thank Mark Sleeman from Monash University Australia for critical readings.

References

1. Mjosberg J, Spits H. Human innate lymphoid cells. *J Allergy Clin Immunol* 2016; 138:1265-76.
2. Morita H, Moro K, Koyasu S. Innate lymphoid cells in allergic and nonallergic inflammation. *J Allergy Clin Immunol* 2016; 138:1253-64.
3. Robinette ML, Colonna M. Immune modules shared by innate lymphoid cells and T cells. *J Allergy Clin Immunol* 2016; 138:1243-51.
4. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat Immunol* 2016; 17:636-45.
5. Everaere L, Ait-Yahia S, Molendi-Coste O, Vorng H, Quemener S, LeVu P, et al. Innate lymphoid cells contribute to allergic airway disease exacerbation by obesity. *J Allergy Clin Immunol* 2016; 138:1309-18 e11.
6. Cols M, Rahman A, Maglione PJ, Garcia-Carmona Y, Simchoni N, Ko HB, et al. Expansion of inflammatory innate lymphoid cells in patients with common variable immune deficiency. *J Allergy Clin Immunol* 2016; 137:1206-15 e1-6.
7. Netea MG, Latz E, Mills KH, O'Neill LA. Innate immune memory: a paradigm shift in understanding host defense. *Nat Immunol* 2015; 16:675-9.

8. Morita H, Arae K, Unno H, Miyauchi K, Toyama S, Nambu A, et al. An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity* 2015; 43:175-86.
9. Glatzer T, Killig M, Meisig J, Ommert I, Luetke-Eversloh M, Babic M, et al. RORgammat(+) innate lymphoid cells acquire a proinflammatory program upon engagement of the activating receptor NKp44. *Immunity* 2013; 38:1223-35.

Figure legend

Fig. 1. Proliferation of ILC-PBMC^{CFSE} co-culture. ILC subsets were cultured with CFSE-labeled autologous PBMC at a ratio of 1:20. The cultures were harvested on day 5 and analyzed by flow cytometry. **A**, FACS plots depicting proliferation of PBMC in the presence of ILC in general. Lymphocytes were gated based on forward and side scatter, and gating out dead cells using a Fixable Viability Dye (eBioscience, Frankfurt, Germany). The region R1 (marked area) depicts the “dividing” (showing CFSE dilution) cells in the presence of ILC. **B**, Of the total viable lymphocytes, the percentages (% in the y-axis) of “dividing” cells were shown for each co-culture. Data derived from 6 blood samples in 4 independent experiments; ILC3 refers to NKp44⁻ ILC3; Statistics by Mann-Whitney Test, * indicates $P < 0.05$, ** indicates $P < 0.01$.

Fig. 2. ILC3 drive CD94⁺ cells expansion. **A**, Representative FACS plots depicting “dividing” cell subsets within PBMC, gating on viable lymphocytes. **B**, Summary of data depicting the proliferation of various cell-types in the presence of ILC. Of each given co-culture, the percentages (% in the y-axis) of various cell types within total viable lymphocytes were shown. **C**, Bar plot depicting the extend of CD94⁺ cell and CD3⁺ T cell expansion in relevance to the parental cell populations, gating on viable lymphocytes: blue bars represent the percentage of proliferating CD94⁺ cells within total CD94⁺ cell; red bars the percentage of proliferating CD3⁺ cells within total CD3⁺ cell. Data derived from 6 blood samples in 4 independent experiments; ILC3 refers to NKp44⁻ ILC3; * $P < 0.05$, Wilcoxon matched-pairs signed rank test; *Error bar* = STDEV.

Capsule Summary:

Using a co-culture model, we discovered that ILC3s negative for natural cytotoxicity receptor NKp44 drive a rapid and dramatic expansion of CD94⁺ natural killer (NK) cells, a data that suggests a novel function of ILC3s.

Key Words:

Innate Lymphoid cell; CD94, nature killer cell;

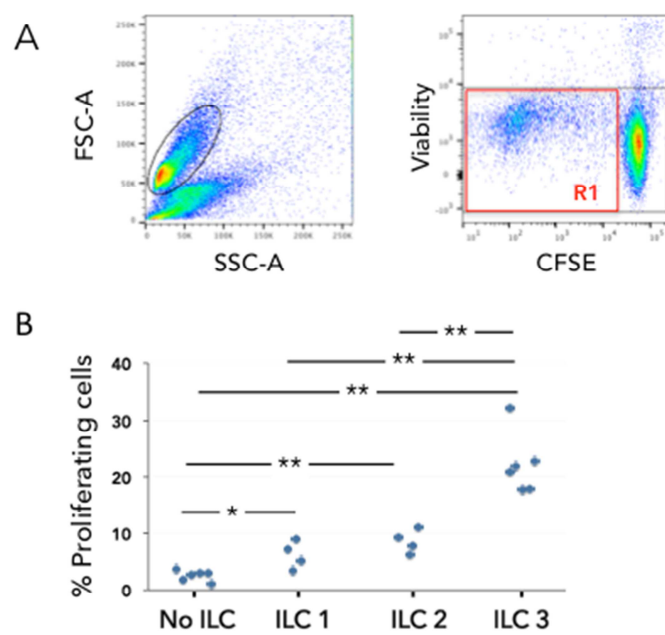


Fig.1. Li et al.

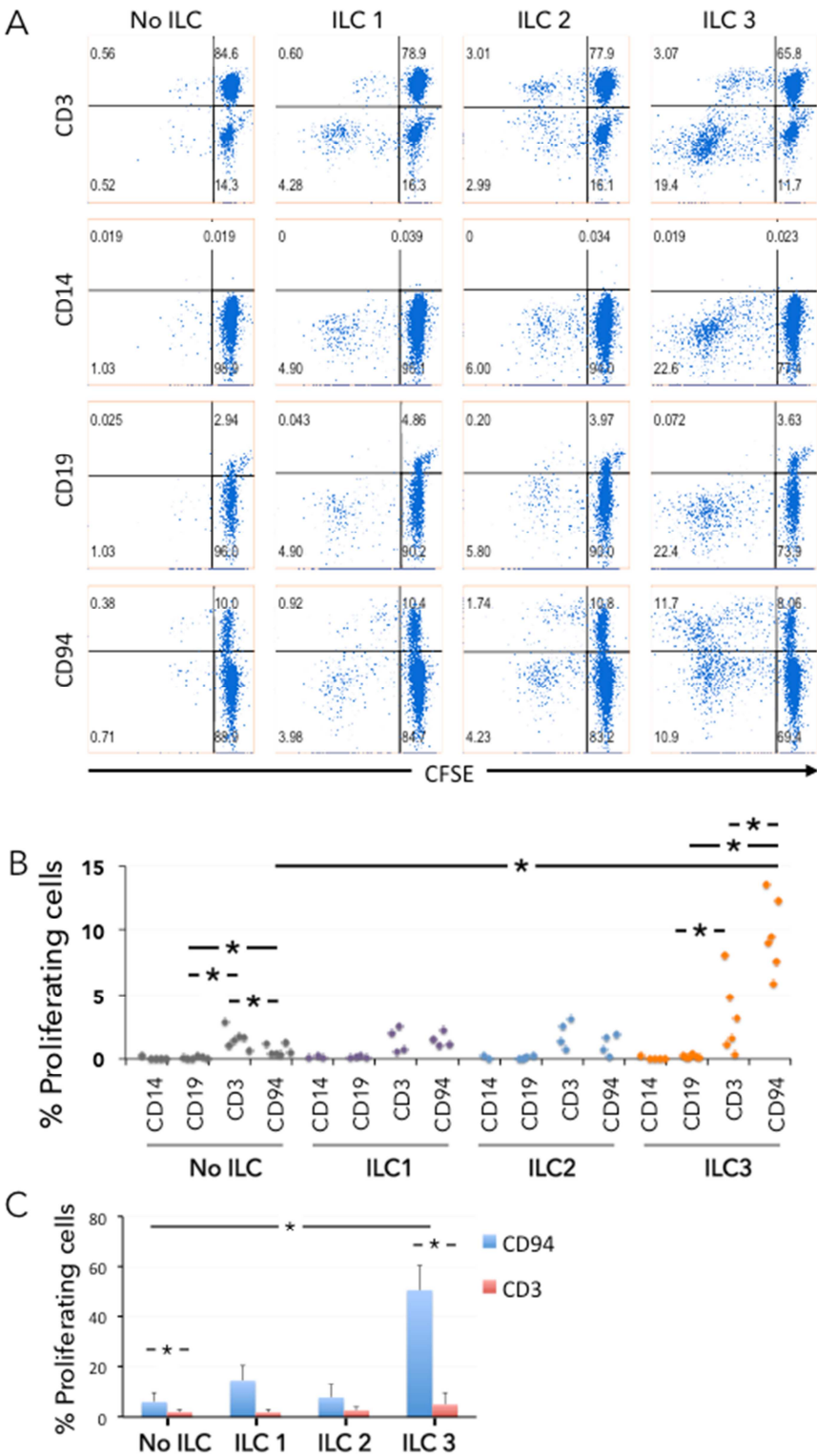
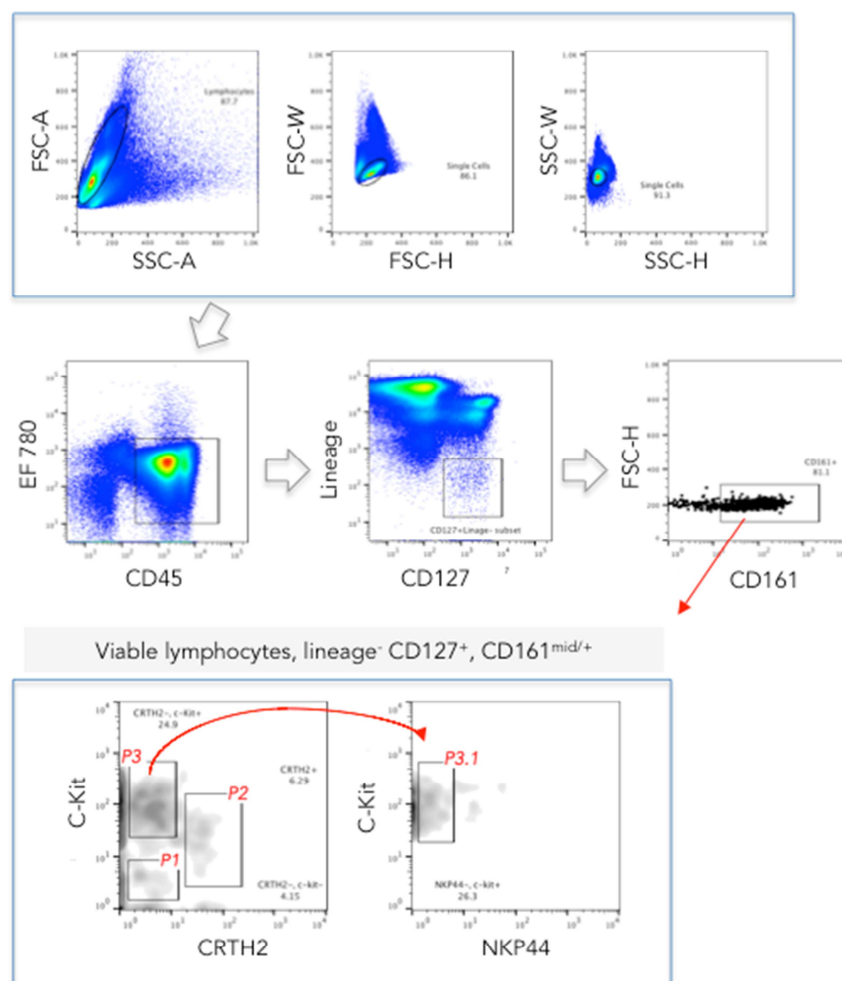
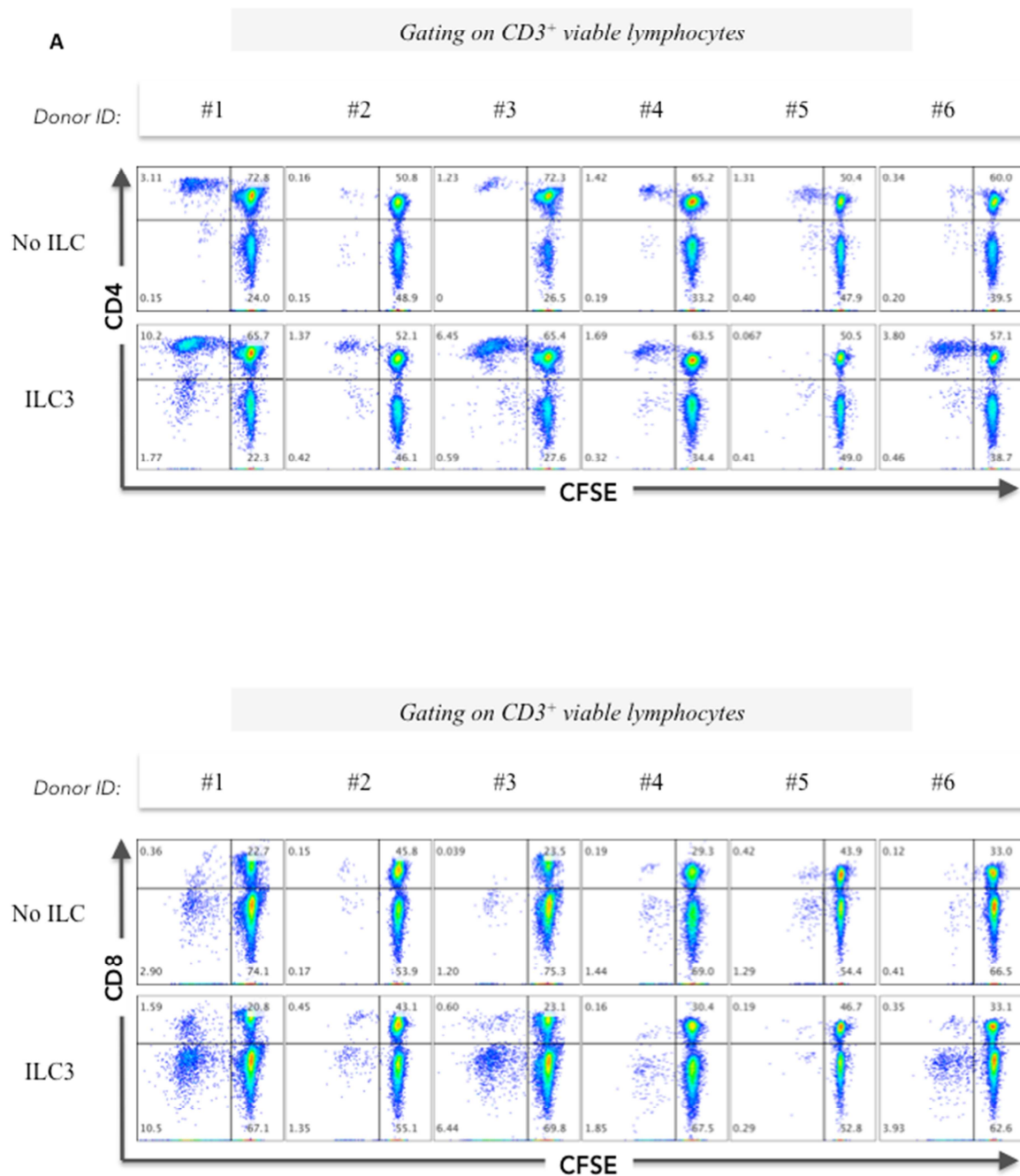
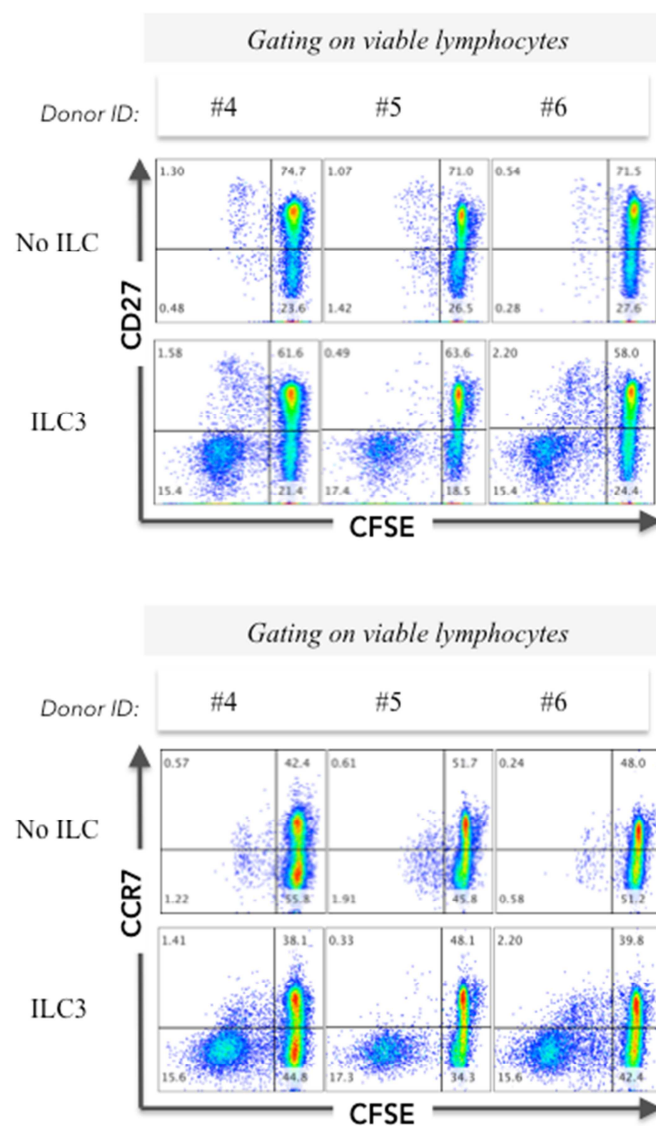


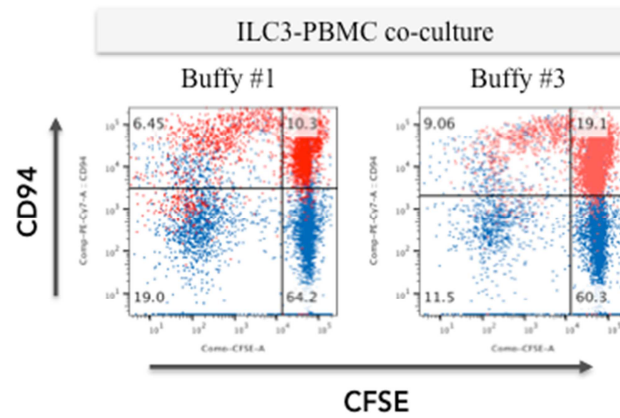
Fig.2. Li et al.





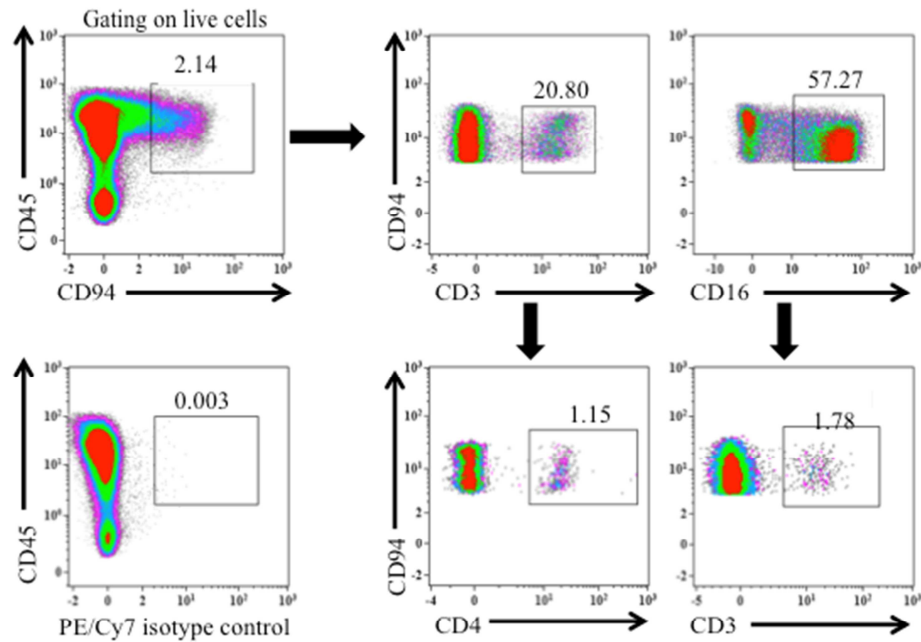
B

c



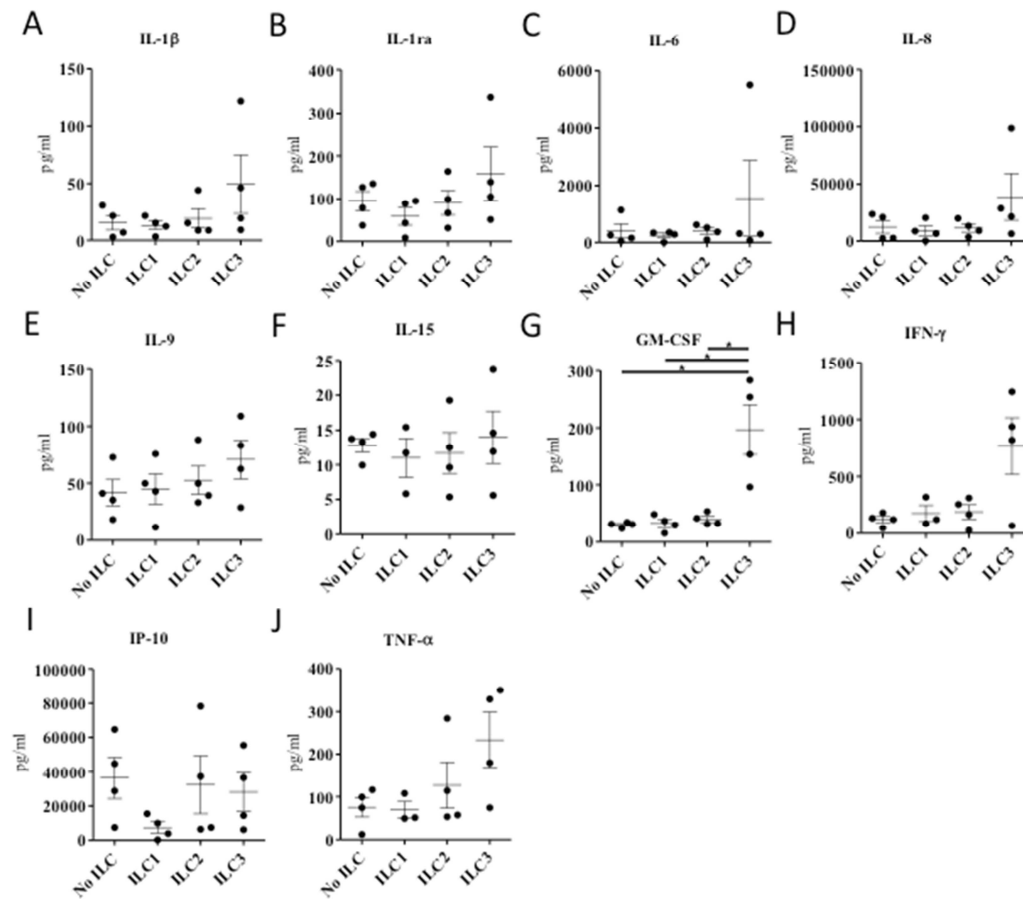
% CD16⁺ (in red) within proliferating CD94⁺ cells, in ILC3-PBMC co-cultures

Buffly #1	35.9
Buffly #2	20.2



Percentage and absolute numbers of CD94 expression on CD3⁺, CD4⁺ and CD16⁺ cells

	% CD45 ⁺ CD94 ⁺ (SD)	# cells (SD)/ μ l
CD45 ⁺ CD3 ⁺ CD94 ⁺	20.80 (8.35)	58.34 (32.19)
CD45 ⁺ CD16 ⁺ CD94 ⁺	57.27 (6.04)	160.81 (19.09)
CD45 ⁺ CD3 ⁺ CD4 ⁺ CD94 ⁺	1.15 (1.03)	4.44 (2.60)
CD45 ⁺ CD3 ⁺ CD16 ⁺ CD94 ⁺	1.78 (1.48)	5.61 (3.94)



Online Repository Fig.4. Li et al

Methods

Isolation of Innate Lymphoid cells: Commercial blood samples from Red Cross were used and the study was approved by Red Cross Oversight Committee. Peripheral blood mononuclear cells (PBMC) were separated using Ficoll-Paque centrifugation. A small proportion of PBMC were kept aside and labelled with CFSE. The remaining PBMC were used for ILC isolation. ILC subsets 1, 2 and 3 were isolated from PBMC as previously described¹ with minor modifications. In brief, CD3, CD14 and CD19 cells were MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) depleted and then sorted using a FACS Aria flow cytometer (BD Bioscience, Allschwil, Switzerland). As outlined in Online Repository Fig. 1, lymphocytes were initially gated based on FSC and SSC, and then dead cells were excluded using a Fixable Viability Dye (eBioscience, Frankfurt, Germany). We then gated on the lineage⁻CD127⁺ viable lymphocytes population, of which the CD161^{mid/+} cells were the main ILC containing population. The three ILC subsets were finally distinguished based on their differential expression of c-kit, CCR2 and NKp44.

ILC-PBMC Co-culture: Freshly isolated ILC as above were mixed with CFSE-labelled autologous PBMC at a ratio of 1:20, a ratio established based on preliminary experiments. The cells were cultured for 5 days, a timing consistent with our previous experiences with short-term cultures^{2,3}, in complete RPMI 1640 media (supplemented with 10% fetal calf serum, 1 mM sodiumpyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin) and incubated at 37°C with 5% CO₂. To encourage cell-to-cell contact, we used 96-w U bottom plate and the cell concentrations were controlled at 1x10⁵ cells / 200µl / well, or as close as possible. The minor cell number variation was caused by insufficient numbers of ILCs in some donors, but the ILC: PBMC ratio was maintained constant.

Flow cytometry analysis: The co-cultures were harvested on day 5, supernatants were kept for cytokine analysis and cells were subjected to flow cytometric analysis. Cells were stained with Fixable Viability Dye and various surface markers, including CD3, CD94, CD14, CD19 and CD16

(antibodies purchased from BioLegend, München, Germany). An initial lymphocyte gate was set based on forward (FSC) and side scatter (SSC), and additional gates were introduced as required. Flow cytometry was performed using BD Aria III, and data were analyzed using FlowJo online software.

Cytokine measurement in supernatants: Co-culture supernatants were analysed for the presence of cytokines using the Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-RAD, Hercules, CA, USA) as per manufactures' instruction. In brief, the standard was reconstituted and diluted in a fourfold dilution series. Culture supernatants 50 µl were measured without dilution. Luminex (Luminex Corporation, Austin, TX, USA) was used to read bead fluorescence and results were analyzed using the xPONENT software (Luminex Corporation).

Statistics: We used non-parametric tests to compare the degree of proliferation in various co-cultures: Mann-Whitney test to compare total lymphocyte proliferation and Wilcoxon matched-pairs signed rank test to compare between different cell types. The software GraphPad Prism was used for this purpose.

References:

1. Morita, H., *et al.* An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity* **43**, 175-186 (2015).
2. Li, S., *et al.* Defining target antigens for CD25+ FOXP3 + IFN-gamma- regulatory T cells in chronic hepatitis C virus infection. *Immunol Cell Biol* **85**, 197-204 (2007).
3. Li, S., *et al.* Analysis of FOXP3+ regulatory T cells that display apparent viral antigen specificity during chronic hepatitis C virus infection. *PLoS Pathog* **5**, e1000707 (2009).

Online Repository Fig. 1: ILC sorting strategy. Lymphocytes were gated based on FSC and SSC, followed by the exclusion of dead cells using Fixable Viability Dye eFluor 780 (eBioscience, Frankfurt, Germany). We focused on the lineage⁻ CD127⁺ viable lymphocytes population, of which the CD161^{mid/+} cells were the main ILC containing cell population. The three ILC subsets were then distinguished based on their differential expression of c-kit, CCR2 and NKp44: P1 is ILC1, P2 is ILC2 and P3.1 is NKp44⁻ ILC3. EF780 stands for Fixable Viability Dye eFluor 780.

Online Repository Fig. 2. Additional characterization of proliferating cells in ILC - PBMC co-cultures. FACS plots depicting **A**, proliferation profiles of CD4 and CD8 T cells are shown gated on viable CD3⁺ lymphocytes; **B**, CD27 and CCR7 expression gated on viable lymphocytes; **C**, the expression of CD16 in co-cultured cells in relevance to CD94 expression, gated on viable lymphocytes. CD16⁺ cells were shown in red dots, and lymphocytes including in blue dots. The missing panels indicate when there was not enough ILC to set up the culture. No ILC and co-culture with ILC3 was used in all experiments. The data demonstrates that there is some CD4⁺ T cell proliferation, but slight, if any, additional proliferation in CD8 T cells, CD27 and CCR7 positive cells over cells without ILC conditions.

Online Repository Fig. 3. Quantification of CD94 expression on ex vivo CD3⁺, CD4⁺ and CD16⁺ cells. Freshly isolated PBMC were stained with Viability dye eFluor 780, followed by staining with fluorochrome conjugated anti-CD3, anti-CD16, anti-CD45 and anti-CD94 mAbs. Cells were gated on live cells (Viability dye eFluor 780⁻) and then gated on CD45⁺CD94⁺ cells. CD45⁺CD3⁺ (T cells) and CD45⁺CD16⁺ (NK cells) were assessed for surface expression of CD94. The dot plot representative gating strategy of CD94 expression on CD3⁺, CD4⁺ and CD16⁺ cell subsets. CD45⁺CD3⁺CD4⁺ (T helper cells) and CD45⁺CD3⁺CD16⁺ (NKT cells) expression on CD94 were gated on CD45⁺CD3⁺CD94⁺ cells and CD45⁺CD16⁺CD94⁺ cells. The numbers in the plot are averaged and represent the percentage of total CD45⁺CD94⁺ cells belonging to each cell subset. Representative data from N = 3 donors.

1 **Online Repository Fig. 4. Cytokine levels in supernatants.** A – J, Cytokine concentrations were
2 measured with a multiplex assay on day 5. Dots represent donor's cytokine measurement and the
3 lines next to the dots indicate standard error of the mean. (N = 3) Comparisons between no ILC,
4 ILC1, ILC2 and ILC3 were performed using Mann-Whitney test. * $p < 0.05$.

5