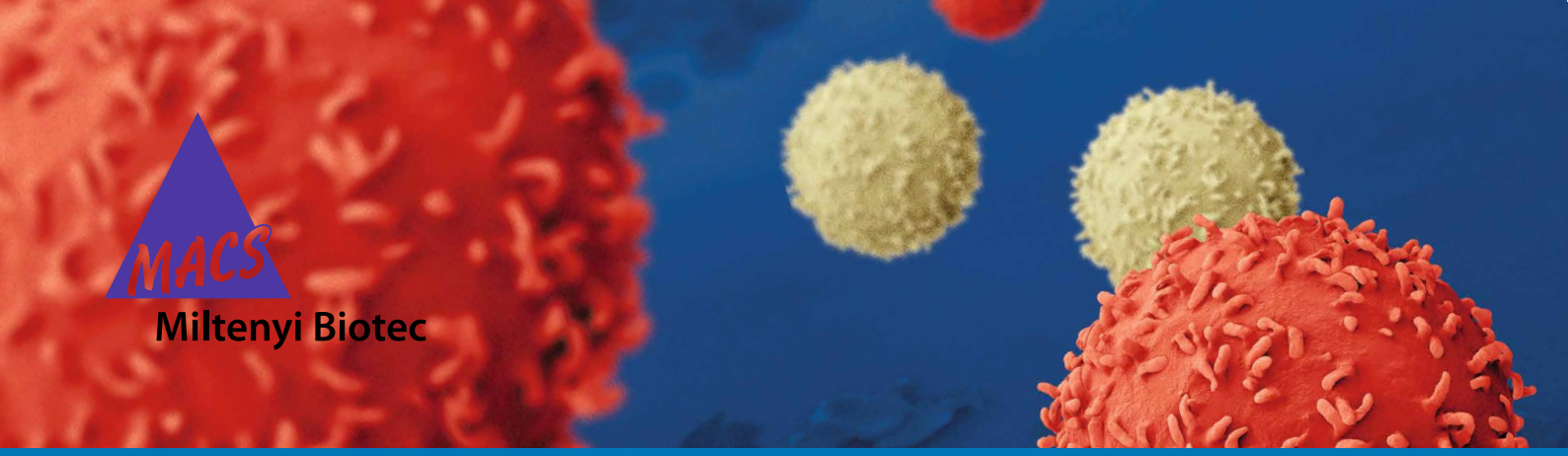

ILC2 isolation from different mouse tissues

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Background

Innate lymphoid cells (ILCs) represent an expanding family of innate effector cells that have crucial roles in the generation and maintenance of immunity, especially at mucosal surfaces[1]. They lack lineage- and rearranged antigen-specific receptors and locate preferentially in non-lymphoid tissues[2]. ILCs are classified in three main groups (ILC1, ILC2, and ILC3) according to their cytokine profile and to the transcription factors required for their differentiation[3].

Since their discovery, the study of ILCs is of ever-increasing interest for the immunology community. In particular, ILC2 cells are important mediators of anti-helminth immunity and in the pathophysiology of allergic inflammation[4], producing large amounts of type 2 T helper cell–associated cytokines such as IL-5, IL-6, IL-9, and IL-13 in response to epithelial cell–derived cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)[3,4].

Most of the gathered knowledge on ILC2 cells results from studies in mice. The phenotypic characterization of mouse ILC2s involves the use of many different surface antigens (KLRG1, Sca-1, c-Kit, Thy-1.2, CD25, and T1/ST2)[3,5]. However, there are a number of discrepancies in the phenotypic characteristics of ILC2s reported in the literature due to the differences in tissue source and preparation methods[5]. The gentleMACS™ Dissociator and MACS® Tissue Dissociation Kits provide the opportunity to standardize the procedure to obtain ILC2 cells from different tissue sources and generate reproducible results. The gentleMACS Dissociator protocol combines automated mechanical tissue dissociation with enzymatic dissociation based on pre-defined enzyme mixes. Thus, in this study, we established an automated tissue dissociation procedure with tailored protocols to obtain optimal ILC2 cells from lung, mesentery, and small intestines to standardize the lab routine.

The protocol below describes the procedure to dissociate mouse lung, mesentery, and small intestine using the gentleMACS Dissociator and specific MACS Tissue Dissociation Kits in order to obtain single-cell suspensions from which viable ILC2 cells can be further isolated by flow sorting and cultured for several weeks. MACS Tissue Dissociation Kits ensure a high viability of ILC2 cells and preservation of epitopes for cell isolation. Furthermore, high-quality cytokines allow reliable cell expansion of ILC2s and REAfinity™ Recombinant Antibodies allow reproducible, background-free flow analysis.

Material and Methods

Tissue dissociation

Lung, mesentery, and small intestine were dissected as previously described[5] from female 9–15 weeks old C57BL/6 mice and dissociated into single-cell suspensions according to the respective dissociation protocols:

Lung lobes were dissected with scissors (no mincing necessary) and dissociated using the Multi Tissue Dissociation Kit 1 in combination with the program 37C_LDK_01 on the gentleMACS™ Octo Dissociator with Heaters. Dissociation solution was prepared in 2.35 mL of serum-free Roswell Park Memorial Institute (RPMI 1640) by adding 100 µL of Enzyme D, 50 µL of Enzyme R, and 12.5 µL of Enzyme A of the Multi Tissue Dissociation Kit 1 into a gentleMACS C Tube.

Fat tissue of mesenteries was dissociated using the Adipose Tissue Dissociation Kit, mouse and rat in combination with the program 37C_mr_ATDK_1 on the gentleMACS Octo Dissociator with Heaters. Dissociation solution was prepared in 2.35 mL RPMI containing 1% bovine serum albumin (BSA) by adding 100 µL of Enzyme D, 50 µL of Enzyme R, and 12.5 µL of Enzyme A of the Adipose Tissue Dissociation Kit, mouse and rat into a gentleMACS C Tube.

Small intestines were dissociated to obtain lamina propria lymphocytes using three different protocols: (1) Epithelial cells and IELs (intraepithelial lymphocytes) were removed as described in the previously published protocol[5], (2)using the Lamina Propria Dissociation Kit, mouse in combination with the gentleMACS™ Octo Dissociator with Heaters following

the complete protocol included in the kit or (3) using the Lamina Propria Dissociation Kit, mouse in combination with the gentleMACS Octo Dissociator with modified conditions in the digestion solution [RPMI containing 2% fetal bovine serum (FBS)].

The resulting single cells from each tissue were suspended in PBS, BSA, and EDTA buffer and the cell number was determined.

Flow analysis of ILC2s

ILC2s were analyzed and sorted following a previously published protocol[5]. In brief, cells were kept cold and pre-cooled solutions were used to prevent capping of antibodies on the cell surface and non-specific cell labeling. PEB buffer, e.g., a solution containing PBS (pH 7.2), 0.5% BSA, and 2 mM EDTA was prepared by diluting MACS® BSA Stock Solution at a ratio of 1:20 with autoMACS® Rinsing Solution. Cell suspensions were labeled with antibodies according to the corresponding data sheets. Successfull ILC2 analysis was furthermore confirmed using the antibodies listed in table 1 (fig. 1).

Cell culture

Sorted ILC2s from naive mice (mesenteric fat tissue) were expanded and maintained in cell culture as follows: 8×10^3–1×10^4 ILC2s per well were seeded in round-bottomed 96-well plates in 10% (vol/vol) FBS/RPMI complete medium containing 10 ng/mL IL-2 or 10 ng/mL IL-7. Half of the medium was changed every two days.
[**Note:** ILC2s derived from mesenteric fat tissue proliferate with IL-2 only, but ILC2s derived from lung or small intestinal lamina propria require both IL-2 and IL-7 for proliferation.]
Sorted ILC2s from naive mice (mesenteric fat tissue) were stimulated in cell culture as follows: ILC2s were cultured with 10 ng/mL IL-33 or 10 ng/mL IL-2 plus 10 ng/mL IL-25 in 10% (vol/vol) FBS/RPMI complete medium.

Functional analysis of ILC2s

Sorted ILC2s were stimulated as mentioned in the previous section with IL-2, IL-7, IL-2 plus IL-25, or IL-33. At day 4 or 5, supernatant was obtained of wells containing approximately 8×10^3–1×10^4 cells. Concentration of IL-5 and IL-13 was detected via ELISA assay using a microplate absorbance reader.

Cell surface antigens	Clone	Fluorochrome
Lineage depletion cocktail	Various*	APC
Anti-Sca-1	REA422	VioBright™ FITC
Anti-KLRG1	2F1	PE
CD90.2	30-H12	PerCP-Vio® 700
Anti-Biotin		APC
CD45.2	104-2	APC-Vio 770
FcR Blocking Reagent		
Propidium Iodide Solution		

* CD3ε, CD4, CD8a, CD11c, FcεRIα, NK1.1, CD19, Ter119, CD5, F4/80, Gr-1 (see material tables)

Table 1: Antibody clones and fluorochromes for ILC2 cell flow analysis.

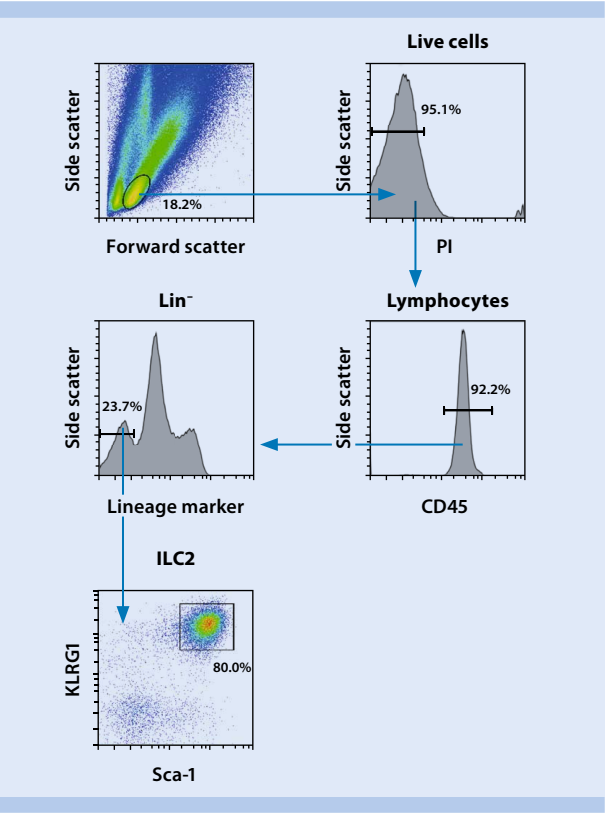

Figure 1: Gating strategy for ILC2 cells from mesenteric fat tissue when using the antibodies mentioned above.

Results

Automated dissociation of various tissues for ILC2 isolation

Single-cell suspensions of lung, mesenteric fat tissue, and small intestine obtained with the gentleMACS™ Octo Dissociator with Heaters and respective MACS® Tissue Dissociation Kits, as described in the material and methods section, contained highly viable ILC2 cells. In comparison to the established protocol, higher numbers of ILC2 cells from small intestine were obtained using the gentleMACS Protocol. With a slight modification of the digestion solution, the cell yield was further increased (fig. 2). Cell surface epitopes were well preserved for labeling with various antibodies against relevant phenotyping markers, as shown by flow cytometry analysis (fig. 3).

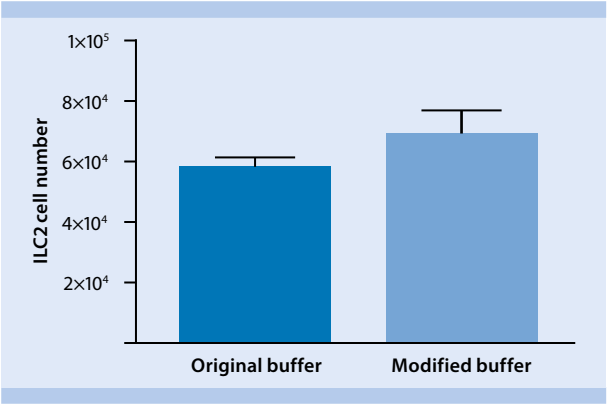

Figure 2: Comparison of ILC2 cell numbers in small intestine after sample preparation using the gentleMACS Dissociator with the Lamina Propria Dissociation Kit with the original vs. modified buffer. The histogram shows the number of ILC2s gated on CD45⁺Lin⁻CD90.2(Thy1.2)⁺Sca-1⁺KLRG1⁺ (n = 2 per group).

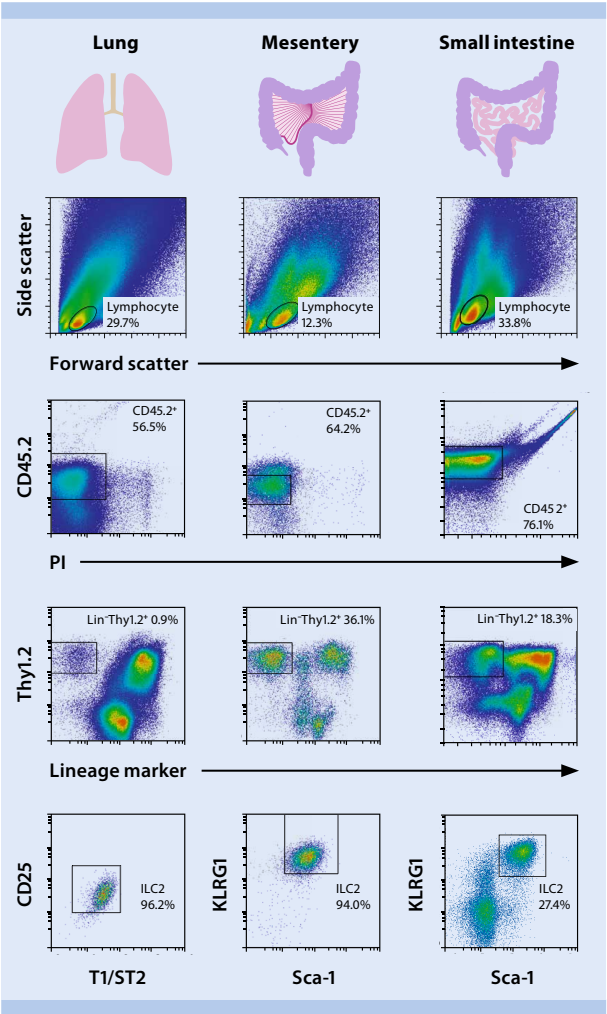

Figure 3: ILC2 cells after dissociation of mouse lung, mesentery, and small intestine with the gentleMACS Octo Dissociator with Heaters and respective MACS Tissue Dissociation Kits. Dissociated cells were stained with the specified antibodies and analyzed by flow cytometry. The ILC2 cells were gated according to the gating strategy described previously[5]: The larger lymphocyte gate was applied based on the forward scatter (FSC) and side scatter (SSC) properties. Doublets were removed to obtain single cells by FSC-W vs. FSC-H and SSC-W vs. SSC-H gating (plots not shown) within the lymphocyte gate. The live leukocytes were then gated on PI⁻ and CD45⁺ and ILC2s were identified by CD90.2 expression (Thy1.2⁺) within the lineage negative cells (Lin⁻) with CD25 and T1/ST2 expression (lung) or Sca-1 and KLRG1 expression (mesentery and small intestine).

Successful expansion of ILC2 cells *in vitro*

Sorted ILC2 cells from mesenteric fat tissue were successfully maintained in culture using either IL-2, IL-7, IL-2 plus IL-25, or IL-33 (fig. 4). As observed in figure 5, IL-7 does not promote ILC2 proliferation but supports ILC2 survival. Successful expansion (cell numbers) is shown in figure 5.

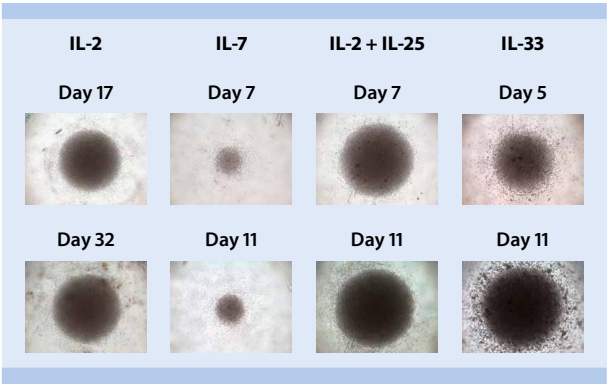

Figure 4: Cell culture of ILC2s isolated from mesenteric fat tissue. Representative images of ILC2s cultured in one well of a round-bottomed 96-well plate at indicated days using 10 ng/mL IL-2, 10 ng/mL IL-7, 10 ng/mL IL-2 plus 10 ng/mL IL-25, or 10 ng/mL IL-33 in 10% (vol/vol) FBS/RPMI complete medium.

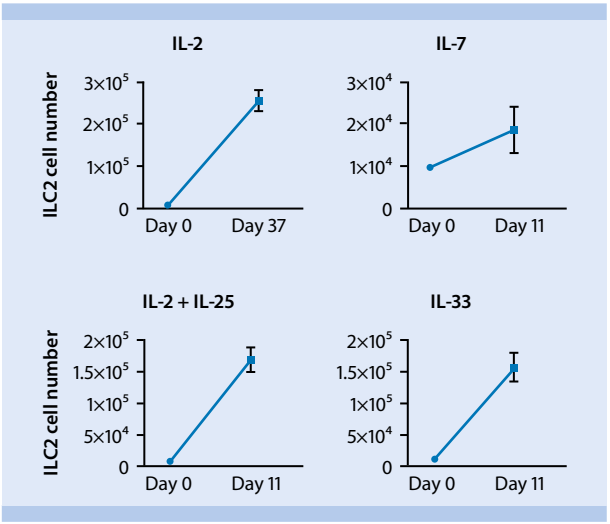

Figure 5: Expansion of ILC2s. 1×10⁴ ILC2s were cultured with different cytokines (using 10 ng/mL IL-2, 10 ng/mL IL-7, 10 ng/mL IL-2 plus 10 ng/mL IL-25, or 10 ng/mL IL-33 in 10% (vol/vol) FBS/RPMI complete medium). ILC2 cell numbers are shown at day 0 and day 11 (IL-7, IL2 + IL-25, and IL-33) or day 37 (IL-2).

Secretion of cytokines from cultured ILC2s

Further analysis of the supernatant of *in vitro* expanded ILC2s isolated from mesenteric fat tissue revealed secretion of IL-5 and IL-13 upon cytokine stimulation (fig. 6).

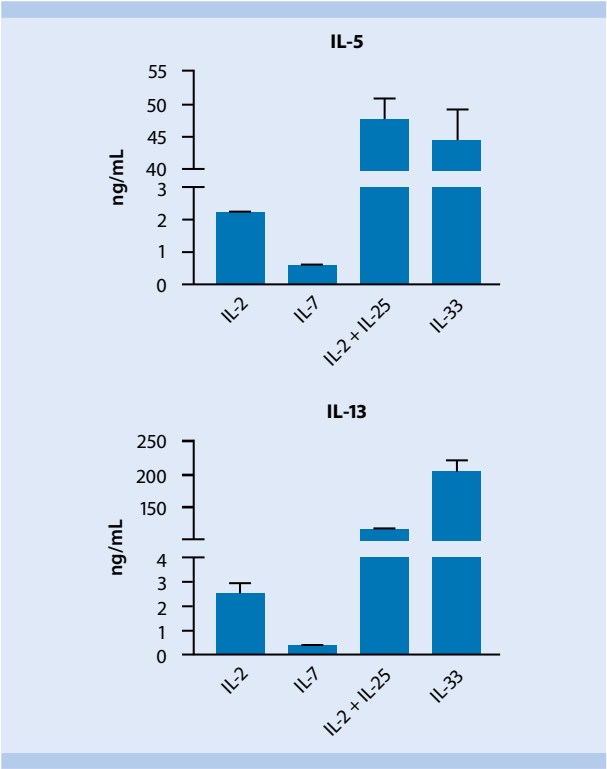

Figure 6: Secretion of IL-5 and IL-13 upon cytokine stimulation. ILC2s were cultured with different cytokines (using 10 ng/mL IL-2, 10 ng/mL IL-7, 10 ng/mL IL-2 plus 10 ng/ml IL-25, or 10 ng/mL IL-33 in 10% (vol/vol) FBS/RPMI complete medium). At day 5 supernatants were obtained and secreted IL-5 and IL-13 was measured using ELISA.

Conclusion

- ILC2s can be successfully isolated using the gentleMACS™ System to obtain high numbers of viable and functional cells
- Standardized, automated tissue dissociation reduces laborious procedures
- Efficient *in vitro* expansion of sorted ILC2 cells can be achieved using highly biologically active recombinant cytokines

References

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4. Moro, K. *et al.* (2010) Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. 63 (7280): 540–544.
5. Moro, K. *et al.* (2015) Isolation and analysis of group 2 innate lymphoid cells in mice. Nature Protocols. 10 (5): 792–806.

Product	Order no.
gentleMACS Octo Dissociator with Heaters	130-096-427
Adipose Tissue Dissociation Kit, mouse and rat	130-105-808
Lamina Propria Dissociation Kit, mouse	130-097-410
Multi Tissue Dissociation Kit 1	130-110-201
C Tubes	130-096-334
MACS SmartStrainers (70 µm)	130-098-462
MACS SmartStrainers (100 µm)	130-098-463
Red Blood Cell Lysis Solution (10×)	130-094-183
MACS Tissue Storage Solution	130-100-008

Product	Order no.
Mouse IL-2, research grade	130-094-054
Mouse IL-7, research grade	130-094-636
Mouse IL-25, research grade	130-115-653
Mouse IL-33, research grade	130-112-958

Product	Clone
CD3ε-Biotin, mouse	17A2
CD4-Biotin, mouse	REA604
CD8a-Biotin, mouse	53-6.7
CD11c-Biotin, mouse	REA754
Anti-FcεRIα-Biotin, mouse	MAR-1
Anti-NK1.1-Biotin, mouse	PK136
CD19-Biotin, mouse	REA749
Anti-Ter-119-Biotin, mouse	REA847
CD5-Biotin, mouse	REA421
Anti-F4/80-Biotin, mouse	REA126
Anti-Gr-1-Biotin, mouse	RB6-8C
Anti-Biotin MicroBeads	–
Anti-Sca-1-VioBright FITC, mouse	REA422
Anti-KLRG1-PE, mouse	2F1
CD90.2-PerCP-Vio700, mouse	30-H12
CD45.2-APC-Vio770, mouse	104-2
Anti-Biotin APC	–
FcR Blocking Reagent, mouse	–
Propidium Iodide Solution	–
7-AAD Staining Solution	–

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