




# Guidelines for mouse and human DC functional assays

**Björn E. Clausen**<sup>#1,2</sup> , **Lukas Amon**<sup>3</sup>, **Ronald A. Backer**<sup>1,2</sup>, **Luciana Berod**<sup>#1,4</sup>, **Tobias Bopp**<sup>1,5</sup>, **Anna Brand**<sup>2</sup>, **Sven Burgdorf**<sup>#6</sup>, **Luxia Chen**<sup>7</sup>, **Meihong Da**<sup>7</sup>, **Ute Distler**<sup>#1,5</sup>, **Regine J. Dress**<sup>8</sup>, **Diana Dudziak**<sup>#3,9,10</sup>, **Charles-Antoine Dutertre**<sup>11,12</sup>, **Christina Eich**<sup>2</sup>, **Anna Gabele**<sup>1,5</sup>, **Melanie Geiger**<sup>13,14</sup>, **Florent Ginhoux**<sup>#11,12,15,16,17</sup>, **Lucila Giusiano**<sup>18</sup>, **Gloria J. Godoy**<sup>18</sup>, **Ahmed E.I. Hamouda**<sup>13,14</sup>, **Lukas Hatscher**<sup>3</sup>, **Lukas Heger**<sup>3</sup>, **Gordon F. Heidkamp**<sup>3</sup>, **Lola C. Hernandez**<sup>19</sup>, **Lukas Jacobi**<sup>3</sup>, **Tomasz Kaszubowski**<sup>3</sup>, **Wan Ting Kong**<sup>11,12</sup>, **Christian H. K. Lehmann**<sup>3,9,10</sup> , **Tamara López-López**<sup>19</sup>, **Karsten Mahnke**<sup>#7</sup>, **Dominik Nitsche**<sup>6</sup>, **Jörg Renkawitz**<sup>#20</sup>, **Rifat A. Reza**<sup>20</sup>, **Pablo J. Sáez**<sup>#19</sup>, **Laura Schlautmann**<sup>6</sup>, **Madeleine T. Schmitt**<sup>20</sup>, **Anna Seichter**<sup>3</sup>, **Malte Sielaff**<sup>1,5</sup>, **Tim Sparwasser**<sup>1,18</sup>, **Patrizia Stoitzner**<sup>21</sup>, **Giorgi Tchitashvili**<sup>3</sup>, **Stefan Tenzer**<sup>#1,5,22</sup>, **Nounagnon R. Tochoedo**<sup>3</sup>, **Damir Vurnek**<sup>3</sup>, **Fabian Zink**<sup>6</sup> and **Thomas Hieronymus**<sup>#13,14,23</sup> 

<sup>1</sup> Research Center for Immunotherapy (FZI), University Medical Center of the Johannes-Gutenberg University Mainz, Mainz, Germany

<sup>2</sup> Institute for Molecular Medicine, Paul Klein Center for Immune Intervention, University Medical Center of the Johannes-Gutenberg-University Mainz, Mainz, Germany

<sup>3</sup> Laboratory of Dendritic Cell Biology, Department of Dermatology, University Hospital Erlangen, Germany

<sup>4</sup> Institute of Molecular Medicine, University Medical Center of the Johannes Gutenberg-University Mainz, Germany

<sup>5</sup> Institute of Immunology, Paul Klein Center for Immune Intervention, University Medical Center of the Johannes-Gutenberg University Mainz, Mainz, Germany

<sup>6</sup> Laboratory of Cellular Immunology, LIMES Institute, University of Bonn, Bonn, Germany

<sup>7</sup> Department of Dermatology, University Hospital Heidelberg, Heidelberg, Germany

<sup>8</sup> Institute of Systems Immunology, Hamburg Center for Translational Immunology (HCTI), University Medical Center Hamburg-Eppendorf, Hamburg, Germany

<sup>9</sup> Medical Immunology Campus Erlangen (MICE), Erlangen, Germany

<sup>10</sup> Deutsches Zentrum Immuntherapie (DZI), Germany

<sup>11</sup> Gustave Roussy Cancer Campus, Villejuif, France

<sup>12</sup> Institut National de la Santé et de la Recherche Médicale (INSERM) U1015, Equipe Labellisée—Ligue Nationale contre le Cancer, Villejuif, France

<sup>13</sup> Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University, Medical Faculty, Aachen, Germany

<sup>14</sup> Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University, Aachen, Germany

<sup>15</sup> Singapore Immunology Network (SIgN), Agency for Science, Technology and Research, Singapore, Singapore

<sup>16</sup> Shanghai Institute of Immunology, Department of Immunology and Microbiology, Shanghai Jiao Tong University School of Medicine, Shanghai, China

<sup>17</sup> Translational Immunology Institute, SingHealth Duke-NUS Academic Medical Centre, Singapore, Singapore

<sup>18</sup> Institute of Medical Microbiology and Hygiene, University Medical Center of the Johannes Gutenberg-University Mainz, Germany

<sup>19</sup> Cell Communication and Migration Laboratory, Institute of Biochemistry and Molecular Cell Biology, Center for Experimental Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

**Correspondence:** Prof. Björn E. Clausen and Dr. Thomas Hieronymus  
e-mail: bclausen@uni-mainz.de; Thomas.Hieronymus@rwth-aachen.de

<sup>#</sup> Section lead authors

<sup>20</sup> Biomedical Center (BMC), Walter Brendel Center of Experimental Medicine, Institute of Cardiovascular Physiology and Pathophysiology, Klinikum der Universität, LMU Munich, Munich, Germany

<sup>21</sup> Department of Dermatology, Venerology & Allergology, Medical University Innsbruck, Innsbruck, Austria

<sup>22</sup> Helmholtz Institute for Translational Oncology Mainz (HI-TRON Mainz), Mainz, Germany

<sup>23</sup> Institute of Cell and Tumor Biology, RWTH Aachen University, Medical Faculty, Germany

This article is part of the Dendritic Cell Guidelines article series, which provides a collection of state-of-the-art protocols for the preparation, phenotype analysis by flow cytometry, generation, fluorescence microscopy, and functional characterization of mouse and human dendritic cells (DC) from lymphoid organs and various non-lymphoid tissues. Recent studies have provided evidence for an increasing number of phenotypically distinct conventional DC (cDC) subsets that on one hand exhibit a certain functional plasticity, but on the other hand are characterized by their tissue- and context-dependent functional specialization. Here, we describe a selection of assays for the functional characterization of mouse and human cDC. The first two protocols illustrate analysis of cDC endocytosis and metabolism, followed by guidelines for transcriptomic and proteomic characterization of cDC populations. Then, a larger group of assays describes the characterization of cDC migration in vitro, ex vivo, and in vivo. The final guidelines measure cDC inflammasome and antigen (cross)-presentation activity. While all protocols were written by experienced scientists who routinely use them in their work, this article was also peer-reviewed by leading experts and approved by all co-authors, making it an essential resource for basic and clinical DC immunologists.

**Keywords:** dendritic cell · endocytosis · langerhans cell · migration · T cell response

## Contents

<b>1 Direct analysis of internalization of proteins (including antibodies) into mouse DC</b>	<b>6</b>
1.1 Introduction	6
1.2 Materials	7
1.2.1 Reagents	7
1.2.2 Equipment	7
1.3 Step-by-step sample preparation	8
1.3.1 Preparation of stocks and solutions	8
1.3.2 Preparation of protein or antibody solution	8
1.3.3 Coupling of the DIBO-SE linker to the protein or antibody of interest	9
1.3.4 Click chemistry reaction of DIBO-SE coupled antibody or protein with the Atto647N-SIP-N3 oligo	9
1.3.5 Titration of the labeled antibody	10
1.3.6 Titration of the labeled BBQ650-quencher	10
1.3.7 Internalization assay	11
1.4 Data analysis	11
1.5 Pitfalls	11
1.6 Top tricks	12
<b>2 Mitochondrial function assessment of mouse Fms-like tyrosine kinase 3 ligand derived-DC using an extracellular flux analyzer</b>	<b>12</b>
2.1 Introduction	12
2.2 Materials	13
2.2.1 Reagents	13
2.2.2 Equipment	13
2.3 Step-by-step sample preparation	14
2.3.1 Generation of FLT3L-DC (FL-DC) cultures from mouse bone marrow	14

2.3.2	Seahorse cell mito stress assay	14
2.3.3	Normalization of Agilent Seahorse XF data by nuclear cell counting using a BioTek Cytation 5	16
2.4	Data analysis	17
2.5	Pitfalls	18
2.6	Top tricks	18
3	Single cell RNA sequencing of human tissue DC	18
3.1	Introduction	18
3.2	Materials	19
3.2.1	Reagents	19
3.2.2	Equipment	19
3.3	Step-by-step sample preparation	20
3.3.1	Preparation of stocks and solutions	20
3.3.2	Index-sorting of human DC	20
3.3.3	Generation of SMARTseq2 single-cell transcriptome data	21
3.4	Data analysis	21
3.4.1	Pre-processing, quality assessment and control, and analysis of SMARTseq2 single-cell transcriptome data	21
3.4.2	Dimensionality reduction	21
3.4.3	Defining clusters	23
3.4.4	Plotting the expression of genes or proteins of interests	23
3.4.5	Integrating indexed-flow cytometry data with scRNAseq data	23
3.5	Pitfalls	23
3.6	Top tricks	23
4	FACS isolation and mass spectrometry-based proteomic analysis of rare mouse DC populations	24
4.1	Introduction	24
4.2	Materials	24
4.2.1	Reagents	24
4.3	Step-by-step sample preparation	26
4.3.1	Single-cell preparation for FACS analysis	26
4.3.2	Flow cytometric purification, collection and lysis of splenic cDC subsets	26
4.3.3	Proteolytic digestion using single-pot solid-phase-enhanced sample preparation (SP3)	28
4.3.4	Liquid chromatography-mass spectrometry (LC-MS)	28
4.4	Data analysis	29
4.5	Pitfalls	30
4.6	Top tricks	32
4.7	Summary table	33
5	Gelatin degradation assay to address podosome formation and function of mouse DC	33
5.1	Introduction	33
5.2	Materials	34
5.2.1	Reagents	34
5.2.2	Equipment	34
5.3	Step-by-step sample preparation	34
5.3.1	Coating of coverslips with fluorescently labeled gelatin	34
5.3.2	Preparation and seeding of DC and performing the gelatin degradation assay	35
5.3.3	Processing cells for podosome detection and fluorescence microscopy	35
5.3.4	Imaging of podosome formation and activity	36
5.4	Data analysis	37
5.5	Pitfalls	38
5.6	Top tricks	38
6	The under-agarose migration assay: a simple method to visualize and quantify mouse DC migration in confined 3D-like microenvironments	38
6.1	Introduction	38
6.2	Materials	39
6.2.1	Reagents	39
6.2.2	Equipment	39
6.3	Step-by-step sample preparation	39

6.3.1	Preparation of the 'under-agarose' assay	39
6.3.2	Injection of DC into the 'under-agarose' assay	41
6.3.3	Live-cell Imaging of DC in the 'under-agarose' assay	41
6.4	<b>Data analysis</b>	41
6.5	<b>Pitfalls</b>	42
6.6	<b>Top tricks</b>	42
7	<b>Quantification of DC migration in 3D collagen matrices</b>	43
7.1	<b>Introduction</b>	43
7.2	<b>Materials</b>	43
7.2.1	Reagents	43
7.2.2	Equipment	43
7.3	<b>Step-by-step sample preparation</b>	44
7.3.1	Generation of bone marrow derived mouse dendritic cells	44
7.3.2	Preparation of collagen gels	44
7.4	<b>Data analysis</b>	45
7.4.1	Analysis of DC migration in collagen (3D environments)	45
7.5	<b>Pitfalls</b>	46
8	<b>Analyzing mouse skin DC migration by whole skin explant culture and FITC painting assays</b>	48
8.1	<b>Introduction</b>	48
8.2	<b>Materials</b>	48
8.2.1	General reagents required for the crawl-out and FITC painting assays	48
8.2.2	General solutions for the crawl-out and FITC painting assays	48
8.2.3	Reagents specific for the crawl-out assay	48
8.2.4	Solutions specific for the crawl-out assay	49
8.2.5	Reagents specific for the FITC painting assay	49
8.2.6	Solutions specific for the FITC painting assay	49
8.2.7	General equipment	49
8.2.8	Equipment specific for the crawl-out assay	49
8.2.9	Equipment specific for the FITC painting assay	49
8.3	<b>Step-by-step sample preparation for the crawl-out assay</b>	49
8.3.1	Preparation of ear tissue	49
8.3.2	Harvesting cells after 24h and 48h	50
8.4	<b>Step-by-step sample preparation for the FITC painting assay</b>	50
8.4.1	Applying FITC reagent mix on mouse ear skin	50
8.4.2	Single-cell suspension of lymph nodes	50
8.5	<b>General flow cytometry staining</b>	51
8.6	<b>Data analysis &amp; gating strategy of migrated ear DC</b>	51
8.7	<b>Pitfalls and top tricks</b>	52
9	<b>Measuring inflammasome activity in human primary DC</b>	54
9.1	<b>Introduction</b>	54
9.2	<b>Materials</b>	54
9.2.1	Reagents	54
9.2.2	Equipment	54
9.3	<b>Step-by-step sample preparation</b>	54
9.3.1	Preparation of stocks and solutions	54
9.3.2	Stimulation of the NLRP3 inflammasome in human DC with ATP	55
9.4	<b>Data analysis</b>	57
9.4.1	Measuring of secreted IL-1 $\beta$	57
9.4.2	Measuring of inflammasome-induced pyroptosis	59
9.4.3	Measuring of active caspase-1	59
9.5	<b>Pitfalls</b>	60
9.6	<b>Top tricks</b>	61
9.7	<b>Summary table</b>	62
10	<b>In vitro antigen-presentation assays with murine DC and CD4<sup>+</sup> T cells</b>	62
10.1	<b>Introduction</b>	62



10.1.1	DC as antigen presenting cells	62
10.1.2	The choice of responder T cells	62
10.1.3	The choice of viable or fixed DC	62
<b>10.2</b>	<b>Materials</b>	63
10.2.1	Chemicals and reagents	63
10.2.2	Consumables	63
10.2.3	Devices	63
10.2.4	Buffers and solutions	63
<b>10.3</b>	<b>Step-by-step sample preparation</b>	64
10.3.1	Preparation of single-cell suspensions from lymph nodes and spleen	64
10.3.2	Isolation of CD4 <sup>+</sup> T cells from single-cell suspensions of spleens and lymph nodes (CD4 <sup>+</sup> T cells Isolation Kit, Miltenyi Biotec <sup>1</sup> )	65
10.3.3	Labeling of Cell Proliferation Dye eFluor™ 450	65
10.3.4	Setting up T Cell proliferation assays	65
10.3.5	Staining of CD4 <sup>+</sup> T cells and analysis by flow cytometry	66
<b>10.4</b>	<b>Data analysis</b>	67
<b>11</b>	<b>Quantitative analysis of <i>in vitro</i> cross-presentation by mouse DC</b>	68
<b>11.1</b>	<b>Introduction</b>	68
<b>11.2</b>	<b>Materials</b>	68
11.2.1	Reagents	68
11.2.2	Equipment	68
<b>11.3</b>	<b>Step-by-step sample preparation</b>	68
11.3.1	Preparation of stocks and solutions	68
11.3.2	Generation of bone marrow-derived DC (BM-DC)	69
11.3.3	Quantification of OVA cross-presentation by flow cytometry using the 25-D1.16 antibody	70
11.3.4	Quantification of cross-presentation after co-culture with antigen-specific T cells by ELISA	70
11.3.5	Monitoring proliferation of activated T cells by flow cytometry	71
<b>11.4</b>	<b>Data analysis</b>	71
11.4.1	Analysis of cross-presentation using the 25-D1.16 antibody	72
11.4.2	Analysis of OT-I T cell activation by ELISA	72
11.4.3	Analysis of OT-I T cell proliferation by flow cytometry	72
<b>11.5</b>	<b>Pitfalls</b>	73
11.5.1	Use of inhibitors/small molecules	73
11.5.2	Sensitivity of the 25-D1.16 antibody	74
11.5.3	Calculation of the division index	74
<b>11.6</b>	<b>Top tricks</b>	74
<b>12</b>	<b>In vivo analysis of killing efficacy by CD8<sup>+</sup> T cell responses</b>	74
<b>12.1</b>	<b>Introduction</b>	74
<b>12.2</b>	<b>Materials</b>	74
12.2.1	Reagents	74
12.2.2	Equipment	74
<b>12.3</b>	<b>Step-by-step sample preparation</b>	75
12.3.1	Preparation of stocks and solutions	75
12.3.2	Immunization of mice	76
12.3.3	Preparation of splenocytes	76
12.3.4	CFSE and CTV labeling of target cells	77
12.3.5	Peptide loading of target cells and injection into the immunized mice	77
12.3.6	Analysis of killing efficacy	78
<b>12.4</b>	<b>Data analysis</b>	79
<b>12.5</b>	<b>Pitfalls</b>	79
<b>12.6</b>	<b>Top tricks</b>	80
<b>13</b>	<b>Measuring naïve T cell responses induced by human primary DC</b>	80
<b>13.1</b>	<b>Introduction</b>	80
<b>13.2</b>	<b>Materials</b>	80
13.2.1	Reagents	80

13.2.2 Equipment .....	80
<b>13.3 Step-by-step sample preparation .....</b>	<b>80</b>
13.3.1 Preparation of stocks and solutions .....	80
13.3.2 Measuring naïve T cell responses using mixed leukocyte reactions with primary DC .....	82
<b>13.4 Data analysis .....</b>	<b>85</b>
13.4.1 Analysis of naïve CD4 <sup>+</sup> T cell proliferation, activation, and polarization .....	85
13.4.2 Analysis of naïve CD8 <sup>+</sup> T cell proliferation, activation, and phenotype .....	86
<b>13.5 Pitfalls .....</b>	<b>87</b>
<b>13.6 Top tricks .....</b>	<b>89</b>
<b>13.7 Summary table .....</b>	<b>89</b>
<b>Acknowledgments .....</b>	<b>89</b>
<b>Conflict of interest .....</b>	<b>90</b>
<b>Author contributions .....</b>	<b>90</b>
<b>Data availability statement .....</b>	<b>90</b>
<b>References .....</b>	<b>90</b>
<b>Abbreviations .....</b>	<b>96</b>

## 1 Direct analysis of internalization of proteins (including antibodies) into mouse DC

### 1.1 Introduction

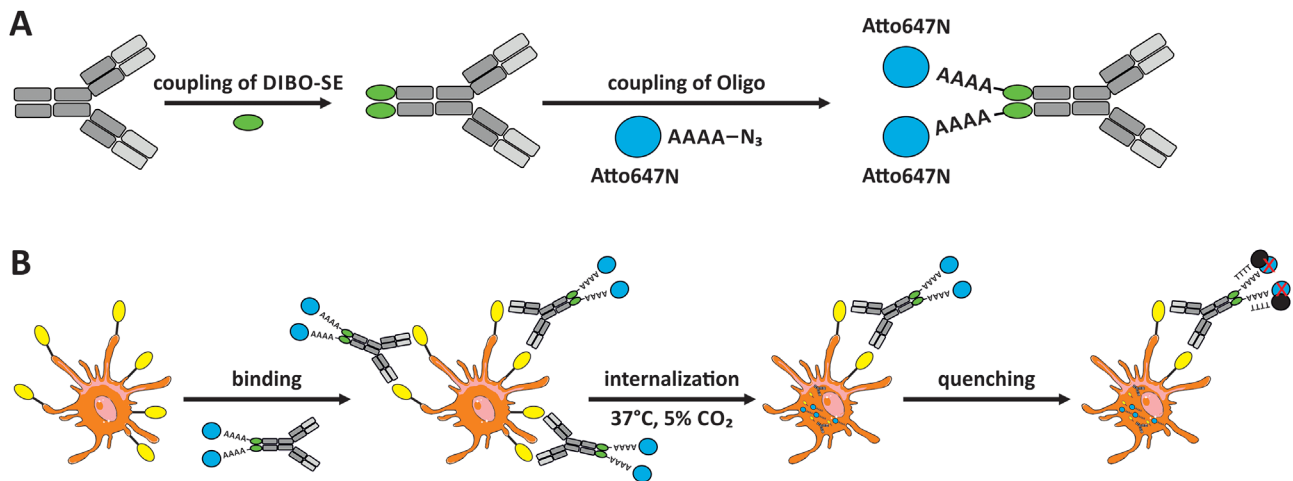
Dendritic cells (DC) belong to the most potent regulators of the adaptive immune response. This includes the induction of new and re-call responses to pathogens, the resolution of inflammation, and the dampening of immune responses to harmless self-antigens [1]. A very important prerequisite for the sentinel function of DC is their ability to uptake material from their surroundings. Therefore, DC can utilize phagocytosis, pinocytosis as well as receptor-mediated endocytosis. Many methods have been proposed allowing to study the uptake of material by DC. These include fluorescently labeled particles and proteins. To specifically assess receptor-mediated endocytosis into DC, different assays have been developed. These include the indirect assessment of endocytosis of antibodies and proteins by staining of the proteins staying outside after a certain incubation time [2–4] and the removal of proteins bound to the outside of the cells, e.g., by acid wash [5, 6]. Both assay formats have major drawbacks: 1) an indirect assay does not allow for the distinction of receptor-mediated endocytosis from the receptor shedding or loss of protein due to a low affinity, while one can also not observe endocytosis over a longer period of time; 2) while the acid wash allows for detection of molecules enriching inside the cell, the acid wash itself, even being only a mild pH change, compromises often many different surface molecules and therefore the different cell subsets might not be clearly identified [5]. An endocytosis assay utilizing directly fluorescent particles or proteins suffers from similar limitations as it is not possible to distinguish just bound particles or proteins from the endocytosed. Alternative, also very useful methods, involve the usage of fluorescent dyes or conjugates which need a pH change (e.g., pHRodo) or a proteolytic degradation to

become fluorescent. However, the here presented method to measure endocytosis does solely focus on internalization and does not require any other step (such as acidification or proteolytic cleavage).

With the here presented method, it is not only possible to observe multiple populations at the same time (e.g., in total splenocytes, in which different cell types may uptake the protein or antibody of interest), but also to directly assess endocytosis and observe this DC function over a longer period. Especially the longer observation period is needed, if it is planned to study receptor-mediated endocytosis mediated by fast recycling receptors, such as Fc receptors [3, 7].

To directly measure endocytosis, a special labeling of the protein or antibody of interest is used (see Fig. 1). Therefore, an Oligo coupled to a fluorescent molecule will be coupled to the protein or antibody to be investigated. After incubation of the cells of interest, such as pure or enriched DC populations or splenic single-cell suspensions, the fluorescence of the proteins or antibodies still bound outside of the cells, will be quenched by using a complementary Oligo labeled with a compatible quencher molecule. This method was adapted from Liu et al., Reuter et al., and Lehmann et al. [3, 5, 8].

This assay works best with freshly isolated, immature DC as maturation often lowers their endocytic capacity. Therefore, it is very important to ensure that no endotoxin is introduced in this assay. Therefore, the reagents have to be tested for endotoxin contamination. We also recommend using the DC (or cell suspensions) directly in this assay to minimize culture time as this also might affect their maturation status and functional capacity. In many cases, DC are also more stable within a complete cell suspension from an organ, such as spleen, so we recommend to avoid cell enrichment, if possible. In principle, any source for DC from any tissue can be used, but it is important to ensure the isolation in an immature state.



**Figure 1.** Graphical representation of the method. A) Graphical visualization for the coupling of the antibody (or protein) used in the endocytosis assay. B) Graphical visualization for the endocytosis assay. Cellular images are provided and adapted from Servier Medical Art (smart.servier.com) and these images are licensed under the Creative Commons Attribution 3.0 Unported License (creativecommons.org/licenses/by/3.0).

## 1.2 Materials

### 1.2.1 Reagents

All reagents are listed in Table 1.

**Table 1.** Reagents, chemicals, and solutions

Reagent	Manufacturer	Ordering number
<b>Chemicals &amp; Solutions</b>		
Pure antibody or protein of interest (important: no azid ions, no glycin, no glycerine, no stabilizer proteins – buffer exchange possible)		
Dulbecco's Phosphate Buffered Saline without calcium and magnesium	Sigma	D8537
RPMI1640 no indicator dye (alternatively RPMI1640 with indicator dye)	Gibco/Life Technologies (Sigma)	11835-063 (R8758)
Fetal Bovine Serum (FCS)	Sigma	F7524
4',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher Scientific	62247
Atto647N-SIP-N <sub>3</sub> -Oligo (Atto647N-5'-TCA GTT CAG GAC CCT CGG CT-3'-N <sub>3</sub> )	Microsynth or IDT	Custom made
BBQ650-Quencher (5'-AGC CGA GGG TCC TGA ACT GA-3'-BBQ650 oligo)	Microsynth or IDT	Custom made
Click-iT® DIBO-succinimidyl ester (DIBO-SE)	Thermo Fisher Scientific	C10414
Alternative: DBCO-NHS ester	Kerafast (via Biozol)	FCC009
ZEBA desalting columns MWCO 7 kDa	Thermo Fisher Scientific	89882
ZEBA desalting columns MWCO 40 kDa	Thermo Fisher Scientific	87766
Water-free DMSO (Dimethylsuloxide)	Thermo Fisher Scientific	D12345
Pluronic® F-127	Thermo Fisher Scientific	P3000MP
L-glutamine (200 mM)	Sigma	G7513-100ML
Penicillin/Streptomycin (10,000 Units/ml, 10,000 µg/ml)	Gibco/Life Technologies	15140-122
1M HEPES	Gibco/Life Technologies	15630-056
β-Mercaptoethanol (cell culture grade, 50 mM in PBS)	PAN Biotech	P07-05100
Water BioPerformance certified (cell culture grade ddH <sub>2</sub> O)	Sigma	W3513-1L

**Table 2.** Necessary equipment

Equipment	Company	Purpose
Centrifuge 'Allegra X-15R'	Beckman-Coulter	Centrifugation of 50 ml tubes, 15 ml tubes, and V-bottom plates
Neubauer counting chamber 0.100 mm; 0.0025 mm <sup>2</sup>	Superior Marienfeld	Cell counting
Corning storage bottle (#430518) and 0.22 μM sterile filter (#431118)	Corning	Sterile filtration and storage of solutions
Sterile bench 'Mars Safety Class 2'	Scanlaf	Performance of all aseptic procedures
LSR Fortessa SORP (#647800)	BD	Flow cytometric analysis of single-cell suspensions
96-well V-bottom plate (651 180)	Greiner bio-one	Sample preparation for flow cytometry
50 ml tubes (#352070)	Falcon	Centrifugation of cell suspensions
15 ml tubes (#188271)	Greiner bio-one	Centrifugation of cell suspensions
Serological pipettes (#606180)	Greiner bio-one	Pipetting
FACS tube (#352008)	Corning	Regular FACS tubes for the acquisition of single-cell suspensions
100 μm filter for 50ml tubes (#542000)	Greiner bio-one	Isolation of single cells from tissues
40 μm filters for 50ml tubes (#542040)	Greiner bio-one	Generation of single-cell suspensions from lymphoid tissues by passive filtration
Pestles (#309658)	BD	Passage of organ material via 100 μm filters
6-well plates (#140675)	Thermo Scientific	Storage of organs & mechanical tissue disruption
Milli-Q Advantage A10	Millipore	On-demand provision of Millipore water (ddH <sub>2</sub> O)
Waterbath	GFL	1086
1.5 ml reaction tubes	Greiner bio-one	616261
2.0 ml reaction tubes	Greiner bio-one	623201

### 1.3 Step-by-step sample preparation

#### 1.3.1 Preparation of stocks and solutions

##### DAPI

Dissolve DAPI 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) in cell culture grade water to create a 1 mg/ml stock solution. Store the solution protected from light at 4°C. Dilute the stock solution 1:10,000 in FACS buffer (PBS+2% FCS) to create a working solution for DAPI staining of cells directly before acquisition at the flow cytometer.

##### FCS

Quickly thaw FCS at 37°C in a water bath. Once completely thawed, incubate for 15 min at 42°C in the water bath to destroy complement activity. Directly filter the warm FCS through a sterile 0.22 μm membrane (Corning #431118) into a sterile storage bottle (Corning #430518) and aliquot into 50 ml portions. Use aseptic techniques during the whole procedure. Aliquoted FCS must be stored at -20°C. Avoid freeze-thaw cycles.

##### FACS buffer

Add 2% FCS (v/v) to Phosphate buffered saline solution (PBS).

##### RPMI+5% FCS (culture medium)

Add 5% FCS (v/v) to RPMI1640 medium without indicator dye. If DC are in the focus of analysis, further supplement with 2 mM L-

glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. Dependent of your cells of interest and the protein or antibody to study, this media formulation can be adapted to your needs.

##### DIBO-SE solution

Solubilize 1 mg of DIBO-SE (MW(DIBO-SE) = 488.5 g/mol) in 1023 μl **water-free DMSO** (final concentration 2 nmol/μl) and store small aliquots (e.g., 2.5 μl) at -20°C. Avoid freeze-thaw-cycles.

##### Atto647N-SIP-N3-Oligo

Solubilize this custom-made Oligo in a concentration of 100 μM in cell culture grade H<sub>2</sub>O and store small aliquots (e.g., 10 μl) at -20°C. Avoid freeze-thaw-cycles.

##### BBQ650-Quencher

Solubilize this custom-made Oligo in a concentration of 100 μM in cell culture grade H<sub>2</sub>O and store small aliquots (e.g., 10 μl) at -20°C. Avoid freeze-thaw-cycles.

#### 1.3.2 Preparation of protein or antibody solution

For the labeling of your protein or antibody of interest, it is important to remove all potentially interfering substances. These include glycine, glycerin, and azide ions as these substances can directly react with succinimidyl ester group of the DIBO-SE linker.

The optimal concentration for labeling of antibodies is 1 mg/ml; other proteins should have the same molarity. In case your protein or antibody concentration is too low, it can be increased by an appropriate volume reduction device. For antibodies, Millipore/Amicon MWCO 50 kDa columns (Amicon® Ultra 4 centrifugal filters, Millipore, UFC805096) might be used. However, this might lead to a loss of antibody or protein of up to 50% due to membrane adsorption. Furthermore, these devices are not sterile and may additionally contain glycerin. The removal of unwanted salts, buffer components and organic compounds can be achieved by buffer exchange columns (here for antibodies or bigger proteins use ZEBA desalting columns MWCO 40 kDa to exchange best for plain PBS). The maximum volume for this buffer exchange column is 100  $\mu$ l.

1. Break the cap at the bottom of the desalting column and insert the column in an appropriate collection tube.
2. Open the cap on top of the column slightly.
3. Centrifuge it for 1 min at 1500  $\times$  g and 4°C.
4. Discard the flow through.
5. Pipet 300  $\mu$ l of PBS onto the column and allow it to sink in.
6. Centrifuge it for 1 min at 1500  $\times$  g and 4°C.
7. Discard the flow through.
8. Pipet 300  $\mu$ l of PBS onto the column and allow it to sink in.
9. Centrifuge it for 1 min at 1500  $\times$  g and 4°C.
10. Discard the flow through.
11. Pipet 300  $\mu$ l of PBS onto the column and allow it to sink in.
12. Centrifuge it for 2 min at 1500  $\times$  g and 4°C.
13. Discard the flow through and the collection tube.
14. Remove any liquid on the outside of the column using a tissue.
15. Place the column in a fresh collection tube.
16. Directly pipet the sample (in not more than 100  $\mu$ l) onto the column and allow it to sink in.
17. Pipet another 15  $\mu$ l of PBS as stacker volume onto the column.
18. Centrifuge it for 2 min at 1500  $\times$  g and 4°C.
19. Flow through of about 100  $\mu$ l contains your antibody or protein in PBS in approximately the same concentration as before buffer exchange.

### 1.3.3 Coupling of the DIBO-SE linker to the protein or antibody of interest

For the coupling process, a tenfold molecular excess of DIBO-SE is used. With its succinimidyl ester group, it can react with primary amines found in the lysine residues of the protein. An IgG molecule displays a molecular weight 150 kg/mol or 150  $\mu$ g/nmol. Therefore, to label 75  $\mu$ g antibody (75  $\mu$ l with 1 mg/ml), which equals 0.5 nmol, 5 nmol DIBO-SE are needed. The stock solution is 2 nmol/ $\mu$ l, so 2.5  $\mu$ l DIBO-SE solution is needed. For other proteins, the volumes have to be adjusted.

20. Transfer 75  $\mu$ l of antibody solution (1 mg/ml, 4°C) into a 1.5 ml reaction tube.

21. Add 2.5  $\mu$ l DIBO-SE solution (2 nmol/ $\mu$ l in water-free DMSO).
22. **Mix immediately and very well.**
23. Incubate the reaction mixture for 2 hours at 4°C.

Now, the excess of DIBO-SE and the unwanted small reaction products need to be removed using a ZEBA desalting column MWCO 7kDa.

24. Break the cap at the bottom of the desalting column and insert the column in an appropriate collection tube.
25. Open the cap on top of the column slightly.
26. Centrifuge it for 1 min at 1500  $\times$  g and 4°C.
27. Discard the flow through.
28. Pipet 300  $\mu$ l of PBS onto the column and allow it to sink in.
29. Centrifuge it for 1 min at 1500  $\times$  g and 4°C.
30. Discard the flow through.
31. Pipet 300  $\mu$ l of PBS onto the column and allow it to sink in.
32. Centrifuge it for 1 min at 1500  $\times$  g and 4°C.
33. Discard the flow through.
34. Pipet 300  $\mu$ l of PBS onto the column and allow it to sink in.
35. Centrifuge it for 2 min at 1500  $\times$  g and 4°C.
36. Discard the flow through and the collection tube.
37. Remove any liquid on the outside of the column using a tissue.
38. Place the column in a fresh collection tube.
39. Directly pipet the sample (in not more than 100  $\mu$ l) onto the column and allow it to sink in.
40. Pipet another 15  $\mu$ l of PBS as stacker volume onto the column.
41. Centrifuge it for 2 min at 1500  $\times$  g and 4°C.
42. Flow through of about 75  $\mu$ l contains your antibody or protein in PBS in approximately the same concentration as before.

### 1.3.4 Click chemistry reaction of DIBO-SE coupled antibody or protein with the Atto647N-SIP-N3 oligo

Next, the alkyne of the attached DIBO-SE linker will be coupled with the azide group of the Atto647N-SIP-N3 oligo. This attaches the fluorophore for detection to your protein or antibody of interest. Therefore, the reaction will be performed with a twofold molecular excess of the oligo (stock: 100  $\mu$ M equals 0.1 nmol/ $\mu$ l). If you start with 75  $\mu$ g of antibody (0.5 nmol), 10  $\mu$ l Atto647N-SIP-N3 oligo (1 nmol of the 100  $\mu$ M stock).

43. If you started with 75  $\mu$ g of antibody (equals 0.5 nmol), add 10  $\mu$ l of Atto647N-SIP-N3 oligo (1 nmol of the 100  $\mu$ M stock solution).
44. **Mix immediately and very well.**
45. Incubate the reaction mixture for 16 hours at 4°C.
46. Top-up the volume to 85  $\mu$ l with PBS.

To remove any unbound oligo, a separation using a ZEBA buffer exchange column MWCO 40 kDa is performed.



47. Break the cap at the bottom of the desalting column and insert the column in an appropriate collection tube.
48. Open the cap on top of the column slightly.
49. Centrifuge it for 1 min at  $1500 \times g$  and  $4^{\circ}\text{C}$ .
50. Discard the flow through.
51. Pipet  $300 \mu\text{l}$  of PBS onto the column and allow it to sink in.
52. Centrifuge it for 1 min at  $1500 \times g$  and  $4^{\circ}\text{C}$ .
53. Discard the flow through.
54. Pipet  $300 \mu\text{l}$  of PBS onto the column and allow it to sink in.
55. Centrifuge it for 1 min at  $1500 \times g$  and  $4^{\circ}\text{C}$ .
56. Discard the flow through.
57. Pipet  $300 \mu\text{l}$  of PBS onto the column and allow it to sink in.
58. Centrifuge it for 2 min at  $1500 \times g$  and  $4^{\circ}\text{C}$ .
59. Discard the flow through and the collection tube.
60. Remove any liquid on the outside of the column using a tissue.
61. Place the column in a fresh collection tube.
62. Directly pipet the sample (in not more than  $100 \mu\text{l}$ ) onto the column and allow it to sink in.
63. Pipet another  $15 \mu\text{l}$  of PBS as stacker volume onto the column.
64. Centrifuge it for 2 min at  $1500 \times g$  and  $4^{\circ}\text{C}$ .
65. Flow through of about  $85 \mu\text{l}$  contains your antibody or protein in PBS. We recommend to fill-up the volume to  $100 \mu\text{l}$  to reach a concentration of about  $0.5 \text{ mg/ml}$ .

### 1.3.5 Titration of the labeled antibody

Before starting with the internalization assay, we recommend to perform a titration of your antibody or protein. Thereby you can maximize your signal and ensure specificity. The titrations are performed independently starting with the fluorescently labeled protein or antibody. Therefore, we recommend to use the volumes and cell numbers that you plan to use in the assays (e.g.,  $50 \mu\text{l}$  of cells in a 96-well plate). As we do not measure internalization yet, it is important to incubate the cells at  $4^{\circ}\text{C}$ , so all media and buffers should be already at  $4^{\circ}\text{C}$  and centrifuges should be pre-cooled.

66. Prepare splenic single-cell suspensions (or single-cell suspension from your organ of interest) by digestion with Collagenase D and DNase I as described in “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9] containing protocols for the generation of DC containing single-cell suspensions from spleen, lymph nodes, and thymus.
67. Re-suspend the cells in the appropriate volume of RPMI+5%FCS medium (e.g., if the internalization into  $5 \times 10^6$  cells in  $50 \mu\text{l}$  is planned, re-suspend the cells in a concentration of  $200 \times 10^6$  cells/ml to seed  $25 \mu\text{l}$ ).
68. Seed  $25 \mu\text{l}$  of the single-cell suspensions in each well ( $5 \times 10^6$  cells/well).
69. Add  $25 \mu\text{l}$  of your Atto647N-SIP-labeled antibody or protein in different concentrations diluted in RPMI+5% FCS.
70. Incubate for 30 min at  $4^{\circ}\text{C}$ .

71. Wash the cells three times with FACS buffer as for a usual staining.
72. Optional: Stain the single-cell suspension with additional antibodies to identify your populations of interest (e.g., DC subsets, see “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9]).
73. Analyze the cells at your flow cytometer of choice (For the Atto647N dye, a classical APC/Alexa647 detection is recommended, e.g.,  $640 \text{ nm}$  laser line and a  $670/14 \text{ nm}$  band pass filter).

### 1.3.6 Titration of the labeled BBQ650-quencher

After identification of the maximum staining for the outside of your cells with the Atto647N-SIP-labeled protein or antibody, a titration of the BBQ650-quencher is needed to ensure complete quenching of the signal caused by labeled protein or antibody outside the cells and to get an idea about the minimum amount of BBQ650-quencher needed. Therefore, the cells are labeled with the amount of labeled antibody, which gives the maximum signal and different amounts of the BBQ650-quencher oligo are tested to ensure complete quenching. As the BBQ650-quencher can only access the cell surface, it is of utmost importance to keep the cells and all solutions at  $4^{\circ}\text{C}$ .

74. Prepare splenic single-cell suspensions (or single-cell suspension from your organ of interest) by digestion with Collagenase D and DNase I as described in “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9].
75. Re-suspend the cells in the appropriate volume of RPMI+5% FCS medium (e.g., if the internalization into  $5 \times 10^6$  cells in  $50 \mu\text{l}$  is planned, re-suspend the cells in a concentration of  $200 \times 10^6$  cells/ml to seed  $25 \mu\text{l}$ ).
76. Seed  $25 \mu\text{l}$  of the single-cell suspensions in each well ( $5 \times 10^6$  cells/well).
77. Add  $25 \mu\text{l}$  of your Atto647N-SIP-labeled antibody or protein with the **previously determined antibody concentration** (from 1.3.5) diluted in RPMI+5% FCS.
78. Incubate for 30 min at  $4^{\circ}\text{C}$ .
79. Wash the cells three times with FACS buffer ( $4^{\circ}\text{C}$ ) as for a usual staining.
80. Add different amounts of the BBQ650-quencher (diluted in cold FACS buffer). The usual needed range is around  $1 \mu\text{M}$  (stock  $100 \mu\text{M}$ ).
81. Optional: Stain the single-cell suspension with additional antibodies to identify your populations of interest (e.g., DC subsets, see “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9]).
82. Analyze the cells at your flow cytometer of choice (For the Atto647N dye, a classical APC/Alexa647 detection is



recommended, e.g., 640 nm laser line and a 670/14 nm band pass filter).

Here, the minimum amount of BBQ650-quencher needed to quench the maximum fluorescence of the Atto647N-SIP-labeled protein or antibody is determined. We recommend for the internalization to use two- to fivefold more of the BBQ650-quencher as determined in this titration (incubation during the internalization later might lead to upregulation or transport of the target of your Atto647N-SIP-labeled protein or antibody to the cell surface. By applying an excess of the BBQ650-quencher, you ensure the highest specificity of the internalization assay.

### 1.3.7 Internalization assay

For this assay, some controls are needed. We at least recommend using a fluorescence-minus-one (FMO) control, a similarly labeled isotype control as well as samples stored at 4°C (without any incubation at 37°C). Additionally, we also recommend to block unwanted Fc/Fc receptor interactions by applying blocking antibodies for FcγRIIB/III (common Fc block, clone 2.4G2) as well as FcγRIV (also known as CD16.2, clone 9E9). As the samples will be incubated for different timespans, we recommend to perform this assay in 1.5 or 2 ml reaction tubes.

83. Prepare splenic single-cell suspensions (or single-cell suspension from your organ of interest) by digestion with Collagenase D and DNase I as described in “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9].
84. Re-suspend the cells in the appropriate volume of RPMI+5%FCS medium (e.g., if the internalization into  $5 \times 10^6$  cells in 50  $\mu$ l is planned, re-suspend the cells in a concentration of  $200 \times 10^6$  cells/ml to seed 25  $\mu$ l).
85. Seed 25  $\mu$ l of the single-cell suspensions in the 1.5 or 2 ml reaction tubes ( $5 \times 10^6$  cells/well).
86. Add 25  $\mu$ l of your Atto647N-SIP-labeled antibody or protein with the two- to tenfold amount of the **previously determined antibody concentration** (from 1.3.5) diluted in RPMI+5% FCS to also be able to observe a “top-up”-effect over time.
87. Incubate the samples for the wanted timespans (e.g., 0, 10, 30, 60, or 120 min; dependent on your receptor) at 37°C and 5% CO<sub>2</sub> (or your preferred culture conditions); non-internalized controls are strictly stored at 4°C.
88. After the end of the incubation, immediately cool down the sample (best by transferring to wet ice and filling up with 10 to 20 times of the starting volume with cold (4°C) FACS buffer.
89. Wash the cells three times with FACS buffer (4°C) as for a usual staining.
90. Add two- to fivefold amount of the BBQ650-quencher as determined in 1.3.5 (diluted in cold FACS buffer).

91. Optional: Stain the single-cell suspensions with additional antibodies to identify your populations of interest (e.g., DC subsets, see “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9]).
92. Analyze the cells at your flow cytometer of choice (For the Atto647N dye, a classical APC/Alexa647 detection is recommended, e.g., 640 nm laser line and a 670/14 nm band pass filter).

### 1.4 Data analysis

To demonstrate the possibilities of this assay, we performed an exemplary internalization assay with three different antibodies (namely DEC205, only found on cDC1, DCIR2, only found on cDC2, and FcγRIV, found on both cDC subsets). Therefore, we investigated the internalization into cDC1 and cDC2 (Fig. 2). The identification of the DC subsets was performed as described in “**Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues**” [9]. FlowJo v10.8 (BD) was used to extract the mean fluorescence intensity values for cDC1 and cDC2 DC. Next, the mean fluorescence intensity of the sample without Atto647N-SIP-labeled antibody was subtracted (giving the  $\Delta$ MFI values). In Figure 2, we compare the change of these  $\Delta$ MFI values over the different incubation periods and also marked the maximum fluorescence intensity of the extracellular staining with the Atto647N-SIP-labeled antibodies (strictly kept on ice and not quenched). Thereby, two different classes of receptors can be determined: 1) DEC205 as well as FcγRIV is internalizing more antibody over time as could be stained extracellularly, if samples were kept on ice only) providing evidence for a replenishment of internalizing receptors by recycling receptors or receptors stored intracellularly and 2) DCIR2 is internalizing less antibody than bound on the cell surface (at least over the 2 hours incubation period).

### 1.5 Pitfalls

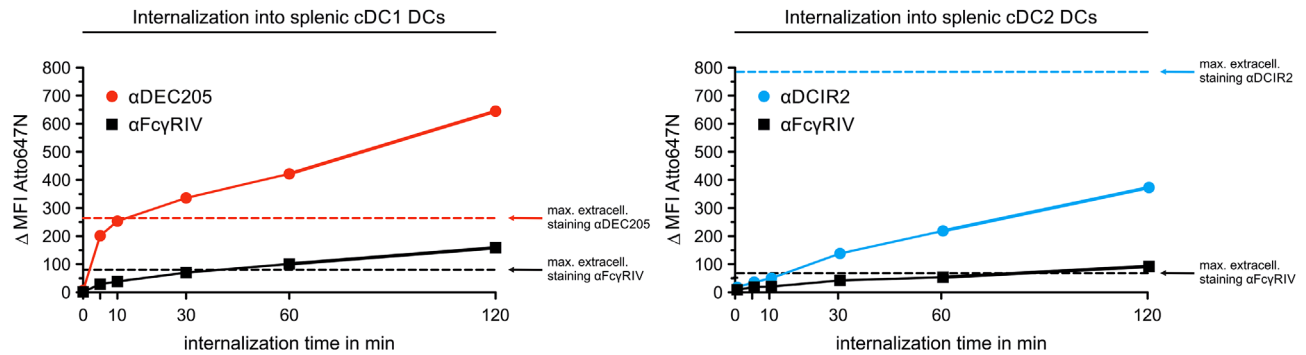
**Problem: No signal from the labeled protein or antibody within the titration.**

**Potential solution:**

Ensure the expression of the antibody target or protein binding partner (e.g., by staining with a classical antibody).

Ensure the correct labeling of your antibody with the DIBOSE and the subsequent Atto647N-SIP-N3 oligo. For this, there are different possibilities. First, for classical antibodies it is possible to use compensation beads, such as UltraComp eBeads™ Plus Compensation Beads (Thermo Fisher Scientific), and ensure, if you can label these beads using the Atto647N-SIP-N3-labeled antibodies. Second, which is also possible for Atto647N-SIP-N3-labeled proteins, the degree of functionalization can be measured on a NanoDrop 1000 UV-VIS spectrophotometer.

Alternatively, also other reactive groups or linkers may be used. These include Click-IT® DIBO-amine (reacting with



**Figure 2.** Exemplary data for the internalization of Atto647N-SIP-labeled antibodies including quenching of extracellular fluorescence. Splenic single-cell suspension of female, ten weeks old C57Bl/6J mice were generated including Collagenase IV/DNase I digestion and erythrocyte lyses. Each  $5 \times 10^6$  cells were incubated with  $10 \mu\text{g/ml}$  Atto647N-SIP- $\alpha\text{DEC205}$ , Atto647N-SIP- $\alpha\text{DCIR2}$ , or Atto647N-SIP- $\alpha\text{Fc}\gamma\text{RIV}$  antibodies in RPMI+5 %FCS for 0, 5, 10, 30, 60, or 120 min at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  and quenched with the BBQ650-quencher oligo. Additional controls included samples without antibody staining as well as an additional sample, which was individually incubated with the same antibody amounts and strictly kept on ice (for the maximum extracellular staining). After staining with the cell identification antibodies, the cells were acquired utilizing a BD LSR Fortessa SORP flow cytometer. Data were analyzed by FlowJo v10.8 (BD) to evaluate the Atto647N fluorescence for cDC1 and cDC2 DC. The panels depict the mean fluorescence intensity for 1) cDC1 (left panel) and 2) cDC2 DC (right panel) in dependency of the internalization period. The dotted lines mark the mean fluorescence intensity of the control sample without internalization and quenching.

carboxylic acids) and Click-iT® DIBO-maleimide (reacting with thiols).

#### Problem: Antibody or protein of interest is not labeled.

##### Potential solution:

Ensure that no other substances including primary amines and no azide groups or ions are present during the reaction with the DIBO-SE linker. Furthermore, ensure that there are enough lysine residues free for the coupling to the DIBO linker in your antibody (normally the case) or your protein. Furthermore, ensure the absence of substances inhibiting the copper-free click chemistry within the reaction with the Atto647N-SIP-N3 oligo.

#### Problem: During the titration of the BBQ650-quencher, only a partial quenching is possible.

##### Potential solution:

Ensure that the sequence of the Atto647N-SIP-N3 oligo is complementary invers to the used BBQ650-quencher. Furthermore, it is important to ensure the right orientation of the BBQ650-quencher to the Atto647N-dye (ensured with the given sequences here), so if the dye is 5', the BB650 BHQ needs to be 3'.

The labeling and the subsequent washing steps were not performed at  $4^\circ\text{C}$  and a partial internalization was taking place. In this case, ensure to keep all buffers, reagents, and centrifuges cold.

The amount of labeled antibody is too high or the amount used quencher is too low. Use less labeled antibody or more quencher.

## 1.6 Top tricks

Here, we describe the analysis of the uptake of one antibody or protein. It can be extended to two or more proteins at the same time. Therefore, you just need to choose a second combination of a DNA sequence, fluorophore, and a compatible quencher.

The labeling technique can be also utilized to label bigger structures, such as nanoparticles or exosomes to analyze their uptake. Furthermore, the Atto647N dye is also compatible to microscopy to visualize the endocytosed antibodies or proteins.

Additionally, the method is in principle also suited to study endocytosis *in vivo*, but high amounts of the conjugate will be needed. We would suspect the need for the injection of 10 to  $100 \mu\text{g}$  per mouse (will strongly depend on the receptor of interest and the antibody or protein used). Shortly after sacrifice, the samples need to be cooled to  $4^\circ\text{C}$  to avoid the continuation of endocytosis before quenching *ex vivo* with the respective quencher.

## 2 Mitochondrial function assessment of mouse Fms-like tyrosine kinase 3 ligand derived-DC using an extracellular flux analyzer

### 2.1 Introduction

In the last years, most research articles interrogating metabolic adaptation of dendritic cells (DC) to microbial stimuli or environmental cues, made use of *in vitro* DC differentiated with granulocyte-monocyte colony-stimulating factor (GM-CSF) [10]. However, the predominant presence of macrophage-like cells and inflammatory DC in this culture setting prevents the extrapolation of results to *bona fide* DC found in physiological conditions [11]. For instance, the presence of other cell types in GM-CSF DC cultures have led to confounding results implying that the sustained upregulation of glycolysis upon Toll-like receptor stimulation, is coupled to DC full activation [12]. However, this could not be demonstrated in other DC cultures lacking inducible nitric oxide synthetase (iNOS) expression or in GM-CSF DC prepared from iNOS KO bone marrows (non-published own data), interrogating

the real contribution of late glycolytic reprogramming to immunogenic DC activation.

To date, the low number of *ex vivo* DC that can be isolated from lymphoid organs does not fulfill the minimum input required for evaluating metabolic processes by conventional methods such as mass spectrometry. Besides, classical biochemical assays that directly measure the consumption of substrates, have become inconvenient and obsolete due mainly to the use of radioactive isotopes.

Therefore, to overcome these limitations, we provide here a protocol for assessing the mitochondrial function of mouse bone marrow-derived DC (BMDC) with an extracellular flux analyzer.

As a model for studying *in vitro* DC metabolism, we propose using the Fms-like tyrosine kinase 3 ligand (FLT3L)-DC culture, which yields a mix of various DC sub-populations (i.e., classical DC1, classical DC2, and plasmacytoid DC) resembling *in vivo* counterparts and thus outperforms the GM-CSF culture in the evaluation of DC immunological and metabolic traits [13].

In addition, the use of the Seahorse XFe96 analyzer to monitor mitochondrial function surpasses the classical methods in several aspects. First, the cell number required is lower than that of other techniques, and multiple conditions can be tested in parallel due to the use of a 96-well microplate. Second and most importantly, it is possible to do real-time measurements of the oxygen consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS), in live cells. Third, the ease of Seahorse's operation and the simplicity and quickness of data analysis turn the Seahorse XFe96 analyzer into a more user-friendly approach than, for instance, mass spectrometry.

Noteworthy, we incorporated a normalization step within the assay workflow based on *in-situ* cell counting with the integrated Cytation 5 multi-mode reader. Data normalization is fundamental during a mitochondrial stress test since OCR heavily correlates to the number of cells per well, and stimulations or treatments can induce either cell proliferation and/or cell death to a variable extent. Albeit protein amount determination upon Seahorse XF analysis has been widely used for the sake of inter-well normalization; it is inaccurate, time-consuming, and does not allow for downstream applications. Cytation 5 fixes these issues by performing rapid automated cell counting of fluorescently-labeled nuclei followed by straight-forward normalization with the Seahorse Wave software.

Our main focus lies on mitochondrial metabolism due to the proven relevance of this organelle for DC differentiation and function [10]. In brief, during the mitochondrial stress test, cells are sequentially treated with compounds that target the electron transport chain (ETC) or disrupt the mitochondrial membrane potential. As a result, various parameters such as the basal respiration and maximal respiration rates can be estimated, and therefore the overall mitochondrial metabolism can be inferred [14].

Finally, gaining accurate knowledge of DC metabolism would contribute to clarifying the role of specific metabolic pathways for DC activation and function, and thereby would enable the phar-

**Table 3.** List of reagents used for Cell Mito Stress assay

Reagent or resource	Company	Catalog
Bone marrow isolation		
Bone marrow cells	-	-
RPMI 1640	Thermo Fisher Scientific	61870
Fetal calf serum (FCS) heat-inactivated	Biochrom	S0115
2-Mercaptoethanol	Gibco	31350
Mouse FLT3L-flag culture supernatant	Made in-house	N/A
Petri dishes 10 cm, sterile	Sarstedt	821473001
50 ml tubes	Sarstedt	62547254
Lipopolysaccharid aus <i>Escherichia coli</i> O111:B4	Sigma Aldrich	L3024
Cell Mito stress assay XFe96 FluxPak including sensor cartridges, cell culture microplates, and calibrant solution	Agilent Technologies	102416-100
XF Seahorse RPMI assay medium, pH 7.4	Agilent Technologies	103576-100
Poly-L-lysine 0.01% solution	Sigma Aldrich	P4707
Sodium Pyruvate 100 mM	Sigma Aldrich	S8636
Glucose	Carl Roth	HN06.2
L-Glutamine	Sigma Aldrich	G3126
Oligomycin	Sigma Aldrich	75351
Antimycin A	Sigma Aldrich	A8674
Carbonyl cyanide-p-trifluoromethoxyphenyl hydrazone (FCCP)	Cayman chemicals	15218
Rotenone	Cayman chemicals	13995
Hoechst 33342 (20 mM)	Thermo Fisher scientific	62249
Ultrapure distilled water	Thermo Fisher scientific	10977-049

macological manipulation of DC metabolism as new therapeutic strategy.

## 2.2 Materials

### 2.2.1 Reagents

A detailed list of necessary reagents for FL-DC generation and Cell Mito Stress assay is provided in Table 3.

### 2.2.2 Equipment

Necessary equipment is listed in Table 4.

**Table 4.** List of necessary equipment

Equipment	Manufacturer	Model
Laminar flow hood	Thermo Fisher Scientific	Herasafe 2030i
Centrifuge	Thermo Fisher Scientific	Megafuge ST Plus Series
Inverted microscope	Zeiss	Primovert
Agilent Seahorse XFe96 analyzer	Agilent technologies	
BioTek Instrument's Cytation 5	Agilent technologies	
Incubation shaker	Infors HT	Minitron
CO <sub>2</sub> incubator	Thermo Fisher Scientific	Heracell 240i

## 2.3 Step-by-step sample preparation

### 2.3.1 Generation of FLT3L-DC (FL-DC) cultures from mouse bone marrow

1. Isolate BM cells from mouse femurs and tibias.
2. Seed  $15 \times 10^6$  BM cells into a 10 cm-dish in a total volume of 10 ml of complete medium supplemented with 15% supernatant of an FLT3L-producing cell line (176 ng/ml) [13].
  - \* Note: Henceforth, complete medium consists of RPMI 1640 supplemented with 2-mercaptoethanol (50  $\mu$ M), penicillin (100 u/ml)/streptomycin (0.1 mg/ml), and FCS (10% v/v). The optimal working concentration of FLT3L needs to be determined empirically upon FLT3L new batch production.
3. Place the dish in a humidified cell incubator (37°C, 5% CO<sub>2</sub>) and culture the FL-DC for 9 days.
4. Harvest the FL-DC on day 9 into a 50 mL tube and centrifuge the cell suspension at 400g at 4°C for 7 min.
  - \* Note: 8–12  $\times 10^6$  cells/dish are expected after 7–9 days of culture
5. Count the cells and resuspend the cell pellet in an appropriate volume of complete medium supplemented with 15% FLT3L supernatant (176 ng/ml).
6. Adjust the concentration to seed 40  $\mu$ l of the cell suspension at a density of  $1 \times 10^5$  FL-DC/well into an XF96 cell culture microplate (see section “seeding the cells”).

### 2.3.2 Seahorse cell mito stress assay

#### Day before assay

Preparing the XF96 cell culture microplate:

1. Coat the XF96 cell culture microplate with 20  $\mu$ l of 0.01% poly-L-lysine (PLL) and incubate in a non-CO<sub>2</sub> incubator for at least 1 h.
2. Carefully, rinse twice the PLL with sterile ultrapure distilled water and dry it under the hood for at least 2 h before seeding cells.

\* Note: The coated cell culture microplate can be stored in the fridge overnight, but make sure to warm it up to RT before using it.

Seeding the cells:

1. Add 40  $\mu$ l/well ( $1 \times 10^5$  FL-DC/well) of the FL-DC cell suspension into a PLL-coated cell culture microplate. Complete by adding 40  $\mu$ l of growth medium (made by complete medium supplemented with 15% FLT3L supernatant (176 ng/ml), LPS (100 ng/ml final concentration in growth medium), or the nitric oxide (NO) donor DETA-NONOate (DETA, 200  $\mu$ g/mL in growth medium). Hence, the final volume per well will be 80  $\mu$ l. An example of a cell plate layout for each condition is shown in **Figure 3**.

\* Note: The addition of FLT3L at the same concentration used for bone marrow differentiation is recommended when overnight stimulation is required (e.g. LPS), as it was shown to increase DC survival.

2. Add 80  $\mu$ l/well of only complete medium supplemented with 15% FLT3L supernatant (176 ng/ml; no cells) into the background wells. In general, at least 4 wells (i.e. 1A, 1H, 12A, 12H) are used as a background/correction well (Fig. 3).
3. Examine the plate under an inverted microscope to ensure the cells are plenty settled at the bottom of the plate.
4. Incubate the cell culture microplate in a 37°C humidified 5% CO<sub>2</sub> incubator for 24 h.

Hydrate cartridge:

1. Open an XFe96 sensor cartridge box and separate the utility plate and sensor cartridge (Fig. 4A).
2. Place the sensor cartridge upside down next to the utility plate and add 200  $\mu$ l/well of sterile XF Calibrant to the utility plate.
  - \* Note: Keep the XF Calibrant solution sterile
3. Put the sensor cartridge back onto the utility plate, submerging the sensors in the calibrant carefully.
4. Place them in a 37°C non-CO<sub>2</sub> incubator overnight. To prevent evaporation of the water, verify that the incubator is properly humidified.

Preparing the Seahorse extracellular analyzer:

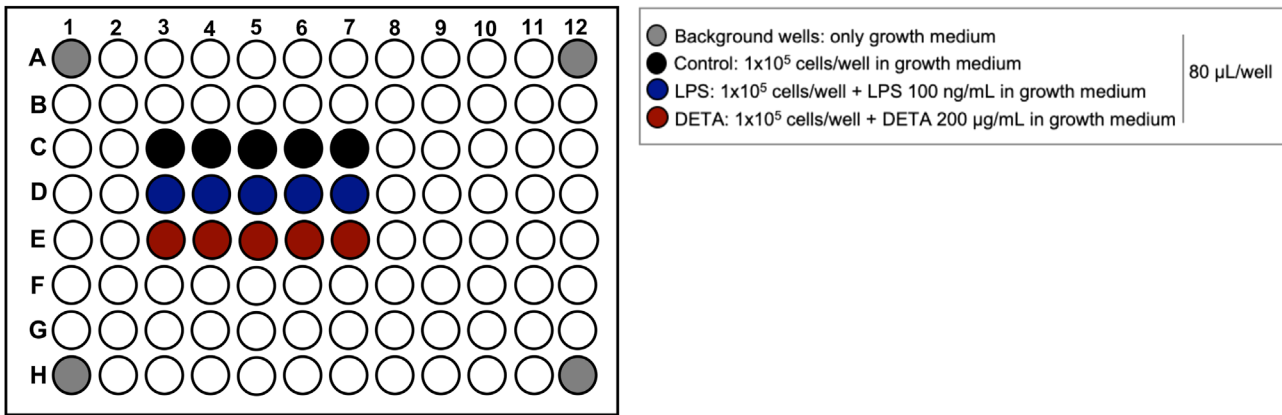
1. Turn on the Agilent Seahorse XFe96 Analyzer, and let it warm up overnight. The analyzer needs to warm up for at least 5 h before starting the assay.
  - \* Note: Make sure the heater is ON and the software recognizes the instrument.

#### Day of assay

Preparing the assay medium and washing the cells:

1. Prepare fresh Mito stress assay medium as shown in Table 5. The final volume depends on how many wells are seeded. We recommend using the Seahorse XF RPMI medium ready to use (low buffered media, pre-adjusted to pH 7.4).



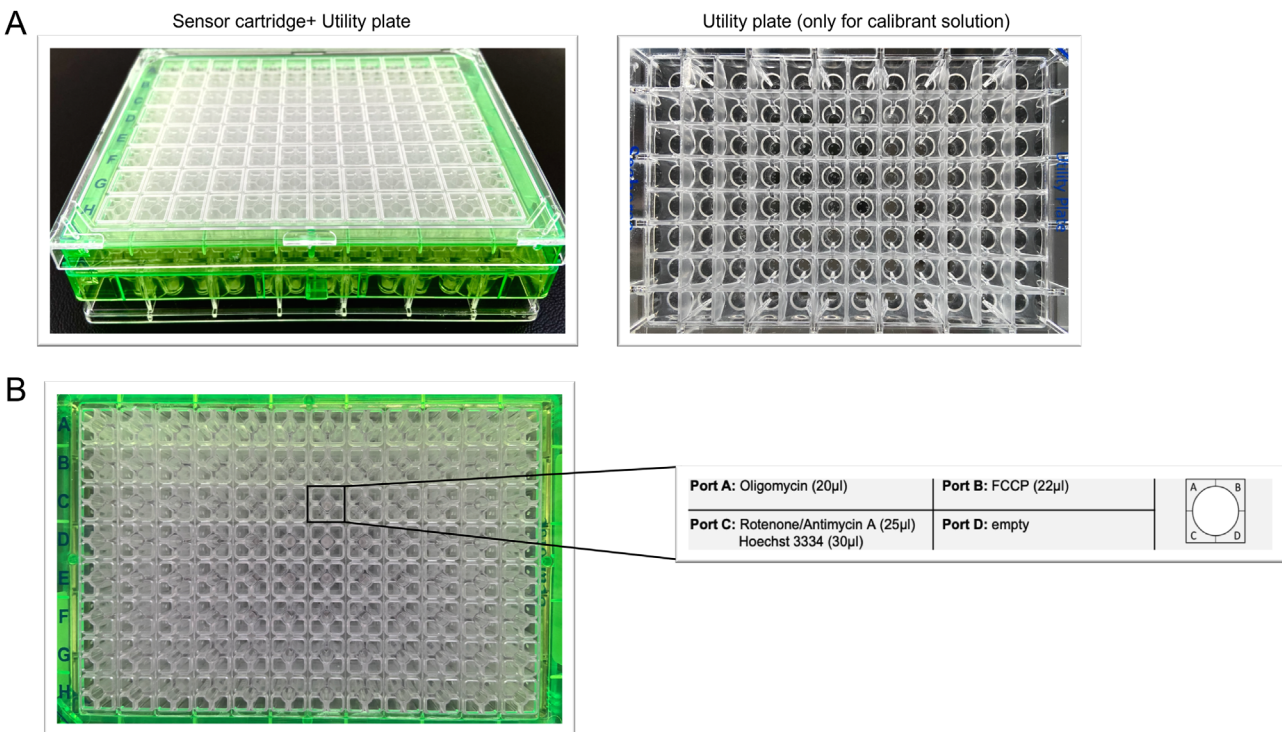


**Figure 3.** Illustration of a cell culture microplate layout for each condition.

- \* Note: The concentration of glucose should also be determined experimentally and adjusted to the cell type of interest.
- Once prepared, warm the XF assay medium in a water bath at 37°C.
  - Retrieve the cell culture microplate from the CO<sub>2</sub> incubator and examine the cells under an inverted microscope to confirm cell health morphology, confluence (uniformity), and purity (no contamination).
  - Remove the XF assay medium from the water bath and wash the cell culture growth medium in the cell culture microplate

by gently adding 180 µl of warmed XF assay medium using a multichannel pipette. Repeat this washing step.

- \* Note: The washing steps should be performed carefully to avoid disrupting the cell monolayer
- Spin down the cell culture microplate slightly in a centrifuge at RT to facilitate the cell adherence to the bottom of the well. Set the acceleration and deceleration to 5 and centrifuge at 250g for 1 min.
  - Examine the cells once again under an inverted microscope to ensure that cells adhered to the bottom of the wells and to verify the presence of consistent monolayer along groups.



**Figure 4.** Photographs of XFe96 sensor cartridge and utility plate. A) Sensor cartridge and utility plate used for an XF Cell Mito Stress assay. B) Representative layout illustrating the corresponding compound to be added into each injection port of the hydrated Sensor Cartridge prior to its calibration.

**Table 5.** XF assay medium

Reagent	Stock	Final concentration	Volume
Seahorse XF RPMI assay medium, pH 7.4 (without phenol red, glucose, pyruvate, and L-glutamine)	-	-	50 ml
Glucose	1 M	2.5 mM	125 $\mu$ l
Pyruvate	100 mM	1 mM	500 $\mu$ l
L-glutamine	200 mM	2 mM	500 $\mu$ l

7. Place the cell culture microplate into a 37°C non-CO<sub>2</sub> incubator for 45 min to 1 h before the assay.

#### Preparation of the mitochondrial modulators and protocol setup

1. Prepare the 10 $\times$  injection solutions for each modulator of mitochondrial respiration using an assay medium. Stocks and final concentrations for each compound are detailed in Table 6. The volume of the solution for each compound suggested is 3 mL for a whole cartridge.
2. Remove the hydrated cartridge from the non-CO<sub>2</sub> incubator and add the appropriate volume of each freshly prepared injection solution into ports A, B, and C as indicated in Figure 4B.
  - \* Note: A nuclear staining dye such as Hoechst 33342 is added on port C in this step to normalize the raw data once the assay is completed (see section "Normalization using a BioTek Instrument's Cytation 5").
3. Inspect the sensor cartridge to verify the solutions are loaded evenly into the ports.
4. Open Wave Desktop software and create a new assay by selecting the template file provided by the manufacturer to

**Table 6.** List of compounds and the corresponding stock and working concentration

Compound	Stock	Final concentration	10 $\times$
Oligomycin	2.5 mM in DMSO	2 $\mu$ M	20 $\mu$ M
FCCP	2.5 mM in DMSO	1.5 $\mu$ M	15 $\mu$ M
Rotenone	2.5 mM in DMSO	0.5 $\mu$ M	5 $\mu$ M
Antimycin A	2.5 mM in DMSO	0.5 $\mu$ M	5 $\mu$ M
Hoechst 33342	20 mM	1.7 $\mu$ M	17 $\mu$ M

**Table 7.** Setting the assay protocol

Step	Compound	Cycle	Measurement details	Duration
Calibrate	-	-	-	
Equilibrate	-	-	-	
Baseline	-	3	Mix-3'; Wait-2'; Measure-3'	24'
Inject port A	Oligomycin	3	Mix-3'; Wait-2'; Measure-3'	24'
Inject port B	FCCP	3	Mix-3'; Wait-2'; Measure-3'	24'
Inject port C	Rotenone Antimycin A Hoechst 33342	3	Mix-3'; Wait-2'; Measure-3'	24'

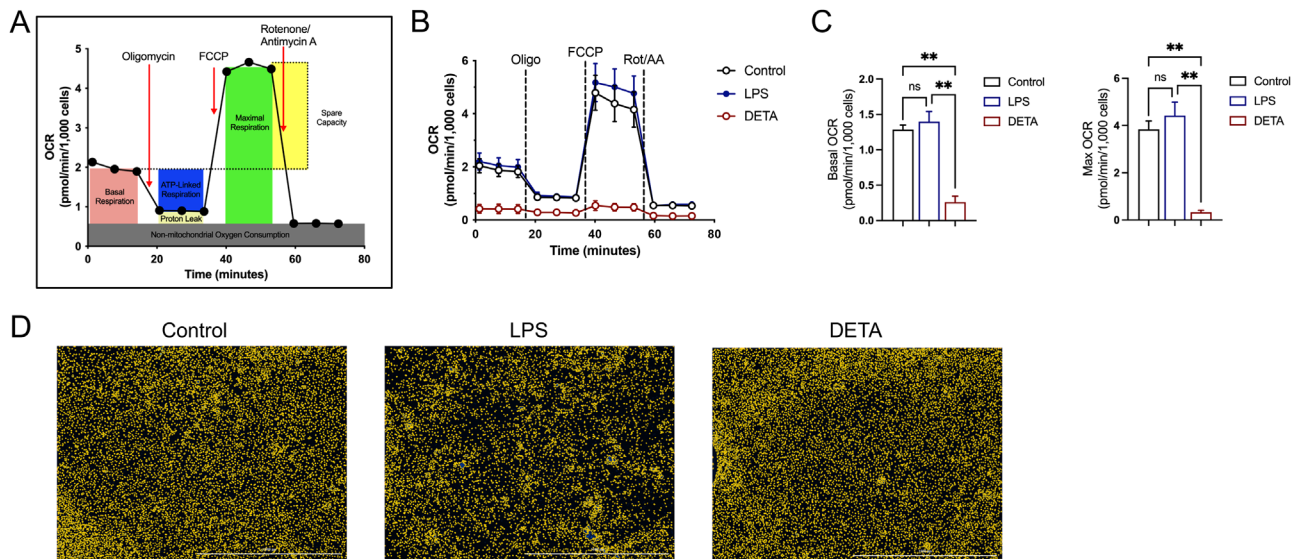
run the XF Cell Mito Stress Test on the Agilent Seahorse XFe96 Analyzer.

5. Set up the standard protocol as shown in Table 7.
6. After setting the conditions for the assay, press the button "Run assay".
7. Follow the instructions on the screen and place the hydrated cartridge without the lid on the Seahorse XFe96 analyzer for calibration and equilibration. The procedure lasts approximately 25 min.
8. Once the calibration is finished, replace the utility plate for the cell culture microplate previously placed in the 37°C non-CO<sub>2</sub> incubator.
9. After the XF Cell Mito stress assay is completed, remove the cell culture microplate and determine the cell counts by using the BioTek Instrument's Cytation 5.
10. Keep the display open to perform normalization of the XF raw data.

#### 2.3.3 Normalization of Agilent Seahorse XF data by nuclear cell counting using a BioTek Cytation 5

1. Turn on the instrument while the XF Cell Mito stress assay is running. The instrument needs time to warm up.
2. Once the XF Cell Mito stress assay is completed, open "Gen5 desktop software" on the Agilent Seahorse XFe96 Analyzer.
3. Transfer the cell culture microplate from the Seahorse XFe96 analyzer to the BioTek Cytation 5 instrument immediately, verifying the microplate is loaded in the correct orientation as indicated in the plate tray.
4. Select "Fluorescent cell counting" on Gen5 Software and choose the necessary wells manually.





**Figure 5.** Metabolic profile of FL-DC upon LPS stimulation. FL-DC were harvested on day 9 and stimulated with 100 ng/ml of LPS or 200  $\mu$ g/mL of DETA in an XFe96 cell culture microplate for 24 h before XF Cell Mito stress assay. **A)** Schematic representation of XF Cell Mito Stress Test profile illustrating the sequential injection of different mitochondrial modulators and the key parameters obtained with this assay. **B)** Oxygen consumption rates (OCR) normalized against cell numbers from FL-DC cultured in the presence or absence of LPS and DETA, measured by extracellular flux analysis. OCR was determined at baseline and after sequential treatment with the mitochondrial complex IV inhibitor oligomycin (Oligo), mitochondrial uncoupler FCCP, and rotenone plus antimycin A (Rot/AA) to inhibit mitochondrial complexes I and III. **C)** Normalized basal OCR (left) and quantification of maximal OCR (Max OCR, right) from FL-DC in the presence or absence of LPS or DETA. Representative results from 2 independent experiments are shown. Data shown are mean  $\pm$  SD,  $n = 5$  technical replicates.  $^{**}P < 0.01$  was determined by one-way ANOVA. **D)** Representative images for each condition obtained by BioTek Cytation5 after cell counting using Hoechst 33342. The nuclear dye was included in the Rotenone/Antimycin A mixture on port C. Nuclear segmentation is represented by yellow contour and was obtained using Gen5 software. Scale bars represent 1000  $\mu$ m.

5. Start the cell counting. The duration of this step depends on the number of wells previously selected. The estimated counting time is approximately 20 min.
6. Once the cell images are captured, remove the cell culture microplate.
7. Inspect the captured images in each well and record them if necessary.
8. Normalize the XF raw data on the Agilent Seahorse XFe96 Analyzer using Wave software.
  - \* Note: As the BioTek Cytation 5 can read the bar code of a specific XF cell culture microplate and both instruments are connected, the cell counting provided will be immediately available on the Seahorse XFe96 analyzer.
9. Proceed to export the Excel or Prism files generated by Wave Software.

## 2.4 Data analysis

This protocol and workflow were validated for FL-DC; however, they could also be used for different cell types exhibiting varied morphological characteristics *in vitro*.

It is highly recommended to check Agilent webpage for more information about the Seahorse XFe96 analyzer before proceeding.

This assay consists of a sequential injection of different compounds that target distinct complexes of the ETC modulating

mitochondrial respiration [15]. Oligomycin, injected first after basal OCR measurements, inhibits the activity of the ATPase (complex V) therefore decreasing OXPHOS (and OCR). FCCP, the second injection, uncouples oxygen consumption from ATP production increasing OCR to the maximum. Therefore, this uncoupler agent estimates the maximum respiration rate that a cell can achieve, afterward used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Spare respiratory capacity is a measure of the capability of the cell to respond to increased energy demand or cellular stress. The third injection consists of a mixture of rotenone and antimycin A, which inhibits complex I and III of the ETC, respectively, and thereby results in a complete OXPHOS blockade enabling the calculation of non-mitochondrial respiration (Fig. 5A).

As previously mentioned, the Seahorse XFe96 analyzer has the advantage of automatically calculating several parameters associated with mitochondrial function from Wave data by using the Cell Mito Stress Test Report Generator.

Although DC metabolism has been widely explored using GM-CSF cultures, few studies have addressed it on conventional DC, either isolated *ex vivo* or from FLT3L-DC cultures. In our setting, we assessed OCR as a measurement of mitochondrial respiration in FL-DC in the presence of LPS or DETA. As shown in Fig. 5B-C, no differences in either basal respiration or maximal oxygen consumption rate (Max OCR) could be detected upon 24h of LPS activation. However, a blockade of the mitochondrial respiration

was observed when FL-DC were stimulated with DETA (Fig. 5C), as previously reported in GM-CSF cultures [16]. In our experimental setting, we utilized bulk FLT3L-DC cultures which consist of a mixture of conventional and plasmacytoid DC. Nevertheless, isolation of individual subpopulations from this DC culture can be performed by magnetic-assisted or fluorescence-assisted cell sorting, resulting then in distinct and cell type-specific mitochondrial respiration profiles.

As we highlighted, the normalization of raw data is an important step to ensure accuracy and consistency in the interpretation of results when performing an extracellular flux analysis. Therefore, we included in our protocol an efficient and simple way to normalize our XF raw data using Hoechst 33342, a membrane-permeant blue fluorescent DNA stain. Combining the *in situ* nuclear staining capability of Seahorse XFe96 analyzer with BioTek Instrument's Cytation 5 system allowed us to normalize our results against cell numbers for all the conditions evaluated. As shown in Fig. 5D, FL-DC were distributed uniformly throughout the wells for all the conditions, however upon LPS activation they appeared disposed in typical aggregates at the bottom of the well.

## 2.5 Pitfalls

The cell seeding density represents a critical step in this assay and must be established beforehand. Excessive number of cells can cause poor cell adhesion and inaccurate OCR measurements. In order to avoid it, it is strongly recommended to examine cell distribution under an inverted microscope to ensure cells are adhered to the coated surface evenly and have minimal cell clusters. If cell loss is detected in a particular well under the microscope, we suggest keeping track of it in case of inconsistency in the results.

Negative OCR values in the calibration step are feasible when trapped bubbles are present between the cartridge and the calibration solution during the hydration of the cartridge.

The appropriate concentration used for each mitochondrial modulator is also a decisive step. In our setting, we have determined experimentally the optimal conditions by titrating each compound in advance (Table 6). If a different cell type will be screened we recommend testing it as the optimal drug concentration varies according to cell type and assay medium (Tables 5 and 6). To note, commonly oligomycin and FCCP concentrations are between 0.5–2.5  $\mu$ M and 0.125–2  $\mu$ M, respectively.

## 2.6 Top tricks

This protocol describes a setting for performing a, XF Cell Mito Stress Test for FL-DC cultures, yet different conditions might be needed for other cell types. Therefore, it is important not only to determine the proper cell density but also the concentration of the mitochondrial modulators experimentally.

Here, we adjusted the optimal cell density at  $1 \times 10^5$  FL-DC/well, however, it varies with different cell types depending mainly on the cell size. In our experience, the optimal cell seeding density usually is between  $1\text{--}2 \times 10^5$  cells/well and should result in cells distributed in the well as a monolayer at 70–90% confluency.

Although we used a PLL-coated cell culture microplate to ensure cell adherence, it is possible to use different plate coating reagents such as Cell Tak, or even ready-to-use poly D-lysine coated XFp Cell Culture microplates provided by Agilent.

When performing the washing steps on the day of assay, it must be done carefully without touching the bottom of the well to prevent loss of cells that might lead to significant dispersion between replicates. Furthermore, we suggest not to remove the total volume existing in the well during this step, especially when using semi-adherent cells. At the end of the washing step, cells must be in a final volume of 180  $\mu$ L, critical to keep the correct concentration of each mitochondrial modulator.

After washing the plate, cells need to be incubated without CO<sub>2</sub> for up to 1 h to ensure a complete attachment to the bottom. However, longer incubation times could substantially affect their viability since XF assay medium has low nutrient concentration.

All ports in the hydrated cartridge need to be filled with the appropriate volume (20, 22, and 25  $\mu$ L for ports A, B and C, respectively), even for those unused. Otherwise, the injection of the wells might be uneven, finally affecting the experimental outcome. In addition, special care must be taken when loading the ports. Owing to the very low volumes pipetted and the small port size, we advise to insert the pipette tip into the port and then dispense the liquid avoiding to create air bubbles. At the end of the port loading process, only when small residual drops are present on top of ports, the cartridge can be gently tapped to settle them to the bottom.

Although all Seahorse XF reagents must be kept sterile, this assay does not necessarily have to be performed in sterility; however, it might be critical for some cell types such as DC.

## 3 Single cell RNA sequencing of human tissue DC

### 3.1 Introduction

Most of our knowledge about dendritic cells (DC), their various subsets, and diverse functions in health and disease came from studies looking at whole cell populations, often by simple microscopy, surface marker flow cytometry analyses, and bulk mRNA sequencing technologies. While this generated very valuable results, allowing us to identify specific characteristics and functions of certain DC subsets and identification of tissue-specific DC populations, these bulk technologies left a lot of questions unanswered. Single-cell analyses and sequencing technologies have since progressed rapidly and single-cell mRNA sequencing (scRNAseq) has become an essential technology performed pretty much on a daily basis, to study cellular phenotypes, functional

**Table 8.** Reagents, chemicals, and solutions

Reagent	Manufacturer	Ordering number
Chemicals & Solutions		
Triton X-100	Sigma-Aldrich	T9284
DNA High Sensitivity Reagent Kit	PerkinElmer	CLS760672
Dulbecco's Phosphate Buffered Saline without calcium and magnesium	Sigma	D8537
EDTA	Promega	V4231
Fetal Bovine Serum (FBS)	Serana	0261S-FBS-SA-015
Illumina Nextera XT kit	Illumina	FC-131-1096
Nextera XT DNA Library Prep (96 Samples)		20027213- 20027216
IDT for Illumina Nextera UD Indexes Set A-D (96 Indexes, 96 Samples)		
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit	Life Technologies	L23105
RPMI 1640	HyClone	SH30255.01
Sodium Azide	Sigma-Aldrich	13412

states, metabolism, and even differentiation pathways of DC in infectious diseases, cancer, autoimmunity, and many other settings [17, 18]. The analyses of single cells vs bulk populations allowed us to identify novel subsets and subset functions, that are easily missed when studying entire tissue populations only.

With the improvement of scRNAseq technologies, these have not just gotten cheaper, with more providers and technologies to choose from [18], but also more accessible to a wider range of researchers. This further led to advances in technologies, such as combining marker-informed sorting of DC populations with scR-

NAseq and even single-cell epigenetic analyses (scATAC-seq), and others. These technologies allow for unbiased and simultaneous characterization of tens of thousands of individual cells, generating massive data sets. This in turn comes with analytical challenges, how to process such large amounts of data, adjust for variability between tissues and species or sparsity of low-represented cell populations, etc. [19–21]. Several computational tools have been developed just over the past few years, facilitating the analyses of scRNAseq data, and new methods are being published constantly. These analyses methods range from data normalization, dimensionality reduction, integration of multiple data sets into one, clustering and visualization of cell populations, differentially expressed genes or differentially expressed gene regulatory networks (regulons), to cell-to-cell interaction analyses, and many more [22–25].

The fast and massive generation of scRNAseq data studying DC across tissues, and species, over the past years, has already improved our understanding of the vast heterogeneity of DC, their specialized functions and roles under physiological conditions, during inflammation, infectious diseases, cancer or autoimmunity, and others. Here, we provide an example for scRNA sequencing of DC, by indexed-SMARTseq2 scRNAseq data of human tissue DC.

## 3.2 Materials

### 3.2.1 Reagents

A list of chemicals and solutions for flow cytometry staining is provided in Table 8. A list of antibodies required is listed in Table 10.

### 3.2.2 Equipment

Necessary equipment is listed in Table 9.

**Table 9.** Necessary equipment

Equipment	Company	Purpose
Centrifuge "Allegra X-15R"	Beckman-Coulter	Centrifugation of 50 ml tubes, 15 ml tubes, and V-bottom plates
Illumina HiSeq 4000	Illumina	Paired-end sequencing
LabChip	Perkin Elmer	Monitoring of length distribution of the cDNA libraries
Sterile bench	-	Performance of all aseptic procedures
96-well V-bottom plate (#GN651180)	Greiner	Sample preparation for flow cytometry
2 ml microcentrifuge tubes (#0030120094)	Eppendorf	Storage and mincing of lung tissue for digest
5 ml polystyrene flow cytometry tubes (#352008)	Falcon™	Staining of samples for flow cytometry
50 ml conical tubes (#352070)	Falcon™	Centrifugation of cell suspensions
Serological pipettes (e.g., #GN606180)	Greiner	Pipetting
Falcon™ 70 µm Cell Strainer, for 50 ml tubes (#352350)	Corning	Filtration of lung tissue, generation of single-cell suspensions

**Table 10.** Antibodies used for Index-sorting of DC populations

Antibodies	Fluorochrome	Clone	Manufacturer	Ordering number
CADM1	Purified	3E1	MBL	CM0004-3
CD1a	AF700	HI149	Biolegend	300120
CD1c	BV421	L161	Biolegend	331526
CD3 $\epsilon$	BV650	SP34-2	BD Biosciences	563916
CD5	BV711	UCHT2	BD Biosciences	563170
CD11b	Biotin	M1/70	BD Biosciences	5533069
CD14	BV650	M5E2	BD Biosciences	563419
CD16	APC/Cy7	3G8	BD Biosciences	302018
CD19	BV650	SJ25C1	BD Biosciences	563226
CD20	BV650	2H7	BD Biosciences	563780
CD45	V500	HI30	BD Biosciences	560777
CD45RA	FITC	5H9	BD Biosciences	556626
CD88	PE/Cy7	S5/1	Biolegend	344308
CD123	BUV395	7G3	BD Biosciences	564195
CD141	APC	AD5-14H12	Miltenyi	130-090-907
CD163	BUV605	GHI/61	Biolegend	333616
CD169	PE	7-239	BD Biosciences	565248
CD206	PE/CF594	19.2	BD Biosciences	563780
HLA-DR	BV786	L243	Biolegend	307642
Fc $\epsilon$ R1 $\alpha$	PerCP	AER-37 (CRA-1)	Biolegend	334616
Anti-chicken IgY	AF647	-	Jackson	703-606-155
Streptavidin	BUV737	-	BD Biosciences	564293

### 3.3 Step-by-step sample preparation

#### 3.3.1 Preparation of stocks and solutions

##### Lysis Buffer

Lysis buffer is prepared as per the SMARTSeq2 protocol [26]. 0.2% (vol/vol) Triton X-100 + 2 U/ $\mu$ l RNase inhibitor. Store at 4°C for up to 6 months.

##### FBS

Thaw FBS in a water bath, at 37°C. Incubate thawed FBS for 30 min at 56°C in a water bath to inactivate. Working in a sterile bench, filter inactivated FBS through a sterile 0.22  $\mu$ m membrane (Corning #431118) into a sterile storage bottle (Corning #430518) and aliquot into 50 ml tubes. Store aliquots at -20°C.

##### Flow cytometry buffer (FACS buffer):

Add 2% FBS + 2 mM EDTA (solved in PBS) + 0.05% Sodium azide in 1 $\times$  PBS. Store at 4°C for long-term storage or on ice for immediate use and during the experiment.

#### 3.3.2 Index-sorting of human DC

For detailed preparation of single-cell suspension from human tissues we refer to “Chapter IX Mononuclear phagocyte phenotypes” in Cossarizza et al. (2021) [27]. The tissue processing protocols described in Cossarizza et al. (2021) [27] are perfectly suitable for processing of human DC for flow cytometry, as well as for scRNAseq.

#### 1. Either:

- a. Prepare single-cell suspensions from human tissue following the protocol described in “Chapter IX Mononuclear phagocyte phenotypes” in Cossarizza et al. (2021) [27].

#### Or

- b. Thaw previously prepared cells from liquid nitrogen and transfer into RPMI supplemented with 20% FBS.
  - Incubate cells with 1 mg/ml DNase I at 37°C until all cell aggregates disappear (up to 30 min).

2. Incubate cells with LIVE/DEAD<sup>TM</sup> Fixable Blue Dead Cell Stain (1:1000), in PBS, for 30 min at 4°C.
3. Without washing add 5% FBS and incubate cells for 15 min at 4°C, in the dark.
4. Add 2 ml of FACS buffer (if using 96-well plate add 200  $\mu$ l) and centrifuge at 650 G for 2 min, at 4°C.
5. Aspirate the supernatant.
6. Re-suspend the cell pellet in 50  $\mu$ l of antibody cocktail (using the antibody panel provided in Table 10). Incubate for 30 min, at 4°C in the dark.
7. Add 2 ml of FACS buffer (if using 96-well plate add 200  $\mu$ l), and centrifuge at 650 G for 2 min, at 4°C.
8. Aspirate the supernatant.
9. *Optional:* If you chose to include CADM1 into your staining (to target cDC1) and since a purified antibody is used to stain for CADM1 on DCs, you will need to perform an additional staining step (otherwise proceed with step 14.):
  - a. Re-suspend the cell pellet in 50  $\mu$ l of FACS buffer containing anti-Chicken-IgY-Alexa-Fluor 647 (to target CADM1). Incubate for 15 min, at 4°C. Then add 2 ml of FACS buffer



(if using 96-well plate add 200  $\mu$ l) and centrifuge at 650 G for 2 min, at 4°C.

b. Aspirate the supernatant.

10. Re-suspend the cell pellet in 200–400  $\mu$ l of FACS buffer, filter through a 70  $\mu$ m cell strainer into a new (clean) 5 ml polystyrene FACS tube, and analyze using a suitable flow cytometer.

For indexed-sorting, cells were recorded and sorted using an ARIAIII 5L (70  $\mu$ m nozzle; BD Biosciences). Fcs files were exported and analyzed using FlowJo v10.8.1 (BD Biosciences).

### 3.3.3 Generation of SMARTseq2 single-cell transcriptome data

Independent of the sequencing method chosen, the basic processing steps are shared across platforms and include 1. cell isolation, followed by 2. cell lysis to access the mRNA within, 3. the conversion of mRNA molecules to cDNA, while barcoding each molecule with a unique identifier (UMI; unique cell and molecular barcode), and last 4. pooling of barcoded/labeled molecules, library construction, and sequencing. Here, we provide an example generating index-sorted SMARTseq2 transcriptome data.

1. Index-sort the above prepared and stained cells directly into 96-well plates containing 3  $\mu$ l of lysis buffer (0.2% Triton X-100, Sigma-Aldrich), using a 70  $\mu$ m nozzle (BD FACS ARIAIII 5L (BD Biosciences)).
2. Prepare single cell cDNA library following the SMARTSeq v2 protocol [26], with a few modifications:
  - a. Use Lysis buffer (as above).
  - b. Use 200 pg cDNA with 1/5 reaction of Illumina Nextera XT kit (Illumina, San Diego, CA, USA).
  - c. The length distribution of the cDNA libraries can be monitored using a DNA High Sensitivity Reagent Kit on the Perkin Elmer Labchip (Perkin Elmer, Waltham, MA, USA).
3. All samples are subjected to an indexed paired-end sequencing run of 2 $\times$ 151 cycles on an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA), with 300 samples/lane.

## 3.4 Data analysis

Indexed-flow cytometry data can be analyzed in a similar manner as classical flow cytometry data using FlowJo (BD). For more detailed analyses and for combining the index-information with the SMARTSeq2 sequencing data the following pipeline can be used. Most of the analyses are performed using R and the below-mentioned R packages. These should be installed prior analyses. For first-time users, we recommend practicing with the “Seurat – Guided Tutorial – 2.700 PBMCs” provided by the Satija lab prior

to analysing their own data ([https://satijalab.org/seurat/articles/pbmc3k\\_tutorial.html](https://satijalab.org/seurat/articles/pbmc3k_tutorial.html)).

### 3.4.1 Pre-processing, quality assessment and control, and analysis of SMARTseq2 single-cell transcriptome data

1. Align paired-end raw reads to the human reference genome (GRCh38 version 25 release; Gencode) using the RSEM program version 1.3.0. A step-by-step tutorial for RSEM is available on [https://github.com/bli25/RSEM\\_tutorial](https://github.com/bli25/RSEM_tutorial) [28]. Following the tutorial calculate the “Transcript Per Million read” (TPM) values. These TPM values are then used for downstream analysis.
2. Next, using the Seurat R package (following the “Seurat – Guided Tutorial – 2.700 PBMCs” tutorial) create a Seurat object first, by using the in step 1 generated TPM as input.
3. Run the quality control (QC). QC metrics are then visualized as violin plots. QC has to be assessed individually for each data set. If all cells have passed the QC continue with step 4, otherwise remove cells that failed the QC before moving on.
4. Normalize and scale data using the ‘NormalizeData()’ and ‘ScaleData()’ function from Seurat.
5. As a next step, highly variable genes can be identified and plotted (for cells that passed the QC) using the ‘FindVariableFeatures’ and ‘VariableFeaturePlot’ functions from Seurat, respectively.
6. Perform the principal component analysis (PCA) using the highly variable genes identified in 3.4.1.5.

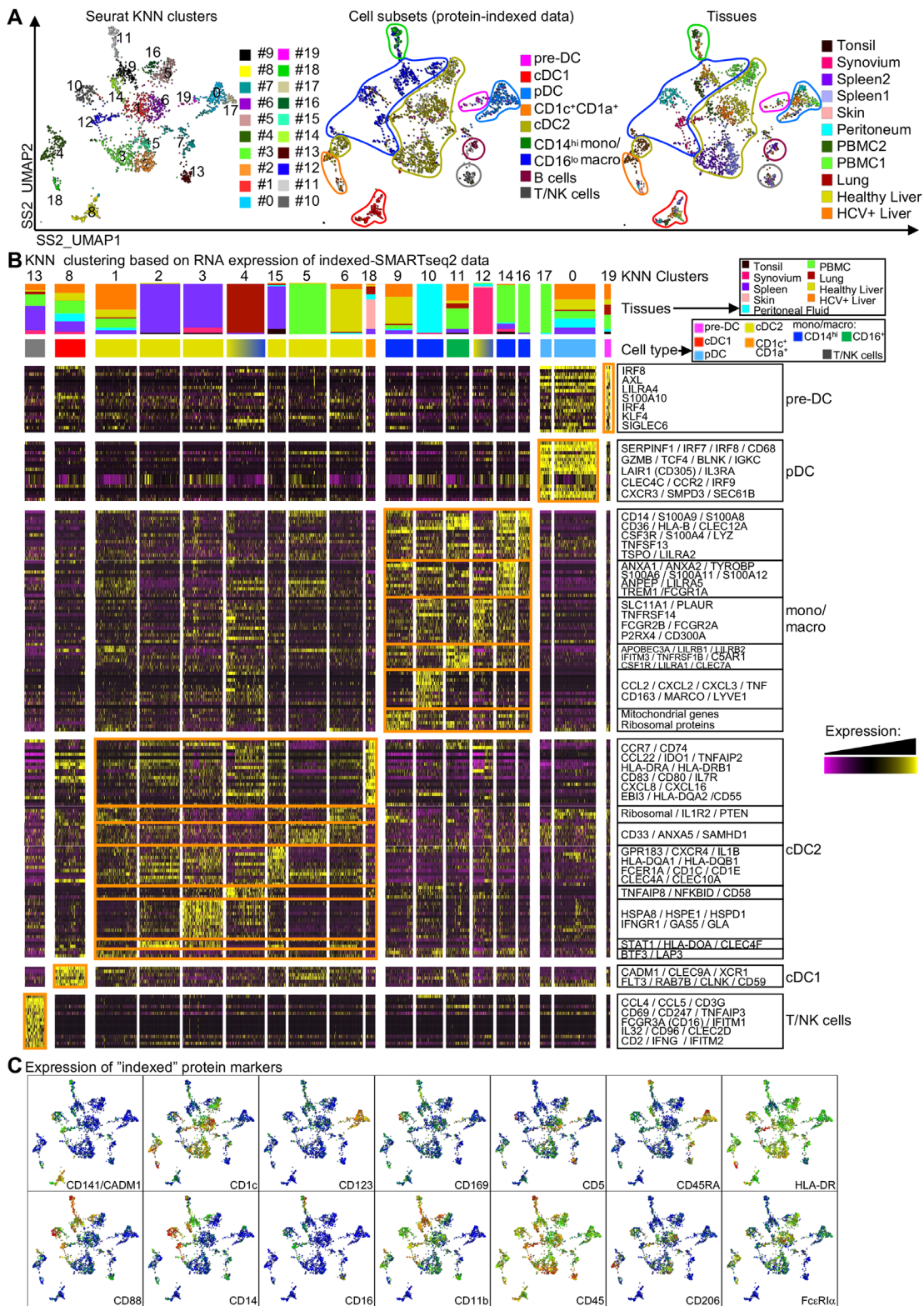
*Note:* All scRNAseq dot plots and meaning plots displaying the gene expression levels or mean signature genes can be generated using R or SeqGeq v1.6 (Flow Jo LLC).

### 3.4.2 Dimensionality reduction

A Uniform Manifold Approximation and Projection (UMAP) [29] of the scRNAseq data will allow us to visualize and explore the datasets in a dimensionally reduced space:

1. To generate such a UMAP use the RunUMAP() function in Seurat using the significant PCs identified previously (in step 6 of 3.4.1 Pre-processing, quality assessment, and control and analysis of SMARTseq2 single-cell transcriptome data), based on elbow/scree plots or Jackstraw obtained from the Seurat analysis.
2. Seurat KNN clustering is implemented for scRNAseq data for an unsupervised classification of cells types (Fig. 6A).

*Note:* To determine an optimal number of clusters for downstream analyses, multiple cluster resolutions are used. Here, Clustree Rpackage (<https://github.com/lazappi/clustree>) is then used to visualize clusters at different resolutions when interrogating clustering.



**Figure 6.** Analyses of indexed-SMARTseq2 scRNAseq data of human tissue DC. (A) Heatmap of differentially expressed genes (DEG) in between SNN clusters (obtained using the Seurat pipeline) of indexed-SMARTseq2 (SS2) scRNAseq data. The relative frequency in each cluster of tissues and cell type annotation is annotated (obtained from DEG and from indexed-data protein expression analysis, not shown). (B) KNN clusters (left panel), cell subsets identification based on indexed-data protein expression analysis (middle panel) and of tissue of origin (right panel) are overlaid onto the UMAP space. (C) Meaning plots showing the relative expression of "indexed" protein markers included in the indexed-SS2 data. (Figure reproduced with permission from Mulder et al., 2021 [30]).



### 3.4.3 Defining clusters

Differentially expressed gene (DEG) analysis is performed to aid cluster annotation. Canonical markers arising from the DEG analysis will allow for matching the unsupervised clustering to known cell types on a transcriptional level (Fig. 6B).

1. Calculate DEGs using normalized values with a logFC threshold of 0.25. Perform a likelihood-ratio test for single-cell gene expression (bimodal test) prior to correction for multiple testing, using the Bonferroni method.
2. Generate and plot a heatmap to visualize e.g., the top 20 DEGs (or all DEGs if these are less than 20) for each cluster.
3. If available, additional metadata (such as tissue identity, DC populations identified using flow cytometry gating of the protein data, etc.) can be overlaid onto the heatmap to ensure robustness of the cell type identity assigned to each cluster here (Fig. 6B).

*Note:* In some cases, closely related clusters could be grouped together to form mega-clusters with biological relevance. For this, the average expression of each gene obtained from the DEG analysis for each Phenograph cluster is derived, to perform a Spearman correlation. A dendrogram heatmap can then be generated using the Ward's method for hierarchical cluster analysis in the pheatmap Rpackage (<https://cran.r-project.org/web/packages/pheatmap/index.html>).

### 3.4.4 Plotting the expression of genes or proteins of interests

Selected genes of interest or mean signature genes can be plotted as meaning plots to display the gene expression levels directly on the UMAP space (for global visualization) or as violin plots (for comparison across different metadata groups/clusters). This can be generated using R or SeqGeq v1.6 (Flow Jo LLC).

1. Using R:
  - a. Visualize individual gene/protein expressions using the FeaturePlot() or VlnPlot() function.
  - b. Calculate a module score using the AddModuleScore function. Use this to visualize the mean expression of a set of gene signatures using FeaturePlot() or VlnPlot().
2. Using SeqGeq,
  - a. Similar to standard flow cytometry data analyses in FlowJo, individual protein expression levels can be visualized using the color axis function.
  - b. To plot mean signatures, select <Genes> → <New Static Gene Stat>
    - i. Select genes (e.g., *XCRI*, *CADMI*, *WDFY4*) and name this list “*cDC1 genes*”
    - ii. On the workspace under <Operations>, select <Mean> and <Apply operations>
    - iii. The mean signature of the list can now be visualized with the color axis function as “*cDC1 genes* <Mean>”

### 3.4.5 Integrating indexed-flow cytometry data with scRNAseq data

Extract the mean fluorescence intensity of the analyzed indexed-flow cytometry from FlowJo. Add these data to the metadata of the Seurat object according to the cell IDs. This allows for the integration of protein information with the scRNAseq analysis for comparative analyses by overlaying protein expression directly onto the UMAP that was generated using transcriptomic analysis (Fig. 6C). This can be done following the steps detailed in 3.4.4.

## 3.5 Pitfalls

Beyond transcriptomic variations in between cells obtained from different patients/donors or different tissues (which correspond to biological variations due to patient-to-patient differences and to different transcriptomic imprinting linked to the variation in tissue microenvironmental signals), a strong bias of scRNAseq data can be due to technical variation in between data obtained in different batches. To circumvent this technical bias, several algorithms (Seurat V4, Harmony, LIGER, and several others) allow to integrated datasets obtained in different batches or using different scRNAseq techniques in a single UMAP or tSNE space.

Depending on the technique employed, the number of transcripts sequenced for each cell varies from 30,000 reads (droplet-based methods such as 10× Genomics Chromium or BD Rhapsody) to up to 1 million reads (SMARTseq2). This only allows to sequence a fraction of the total mRNA pool in each cell and thus, does not allow to detect low copy number transcripts. Furthermore, due to the short half-life of a transcript (much shorter than that of proteins), cells that might have expressed a transcript a few hours before their analysis might not express it anymore at the time of analysis. This phenomenon is called “zero inflation” that can thus be due to biological or technical reasons.

Most immune cell subsets have been identified using protein markers quantified by flow cytometry. With the advent of RNAseq, researchers have observed that there can be a discrepancy in between RNA and protein expression. For example, CD4 is most highly expressed by CD4<sup>+</sup> T cells at the protein level, while the expression of CD4 mRNA is the highest on mononuclear myeloid cells such as monocyte, macrophages, and certain subsets of DC.

## 3.6 Top tricks

The viability of cells and the time spent from tissue harvesting to cell processing to generate the RNA library is critical to avoid technique-related gene expression variations. Human tissue samples should be processed as soon as possible upon reception. Leaving samples overnight prior to processing is not recommended. Ensure that incubation times are strictly followed as per the protocols. Due to the delicate nature of RNA, the workstation should be RNase free and personal protective equipment (e.g., lab coat, gloves, mask) should be worn when processing the samples. We

suggest to work quickly but precisely, keeping cells and RNA chilled at all times possible.

## 4 FACS isolation and mass spectrometry-based proteomic analysis of rare mouse DC populations

### 4.1 Introduction

Acting at the interface of innate and adaptive immunity, dendritic cells (DC) fulfill a multitude of crucial functions in orchestrating and initializing pathogen-specific immune responses. DC can be grouped into two major classes, natural type I interferon-producing plasmacytoid DC (pDC) and conventional (c)DC. The latter can be further divided into phenotypically and functionally distinct subpopulations. While cDC1 (including splenic XCR1<sup>+</sup> CD8 $\alpha$ <sup>+</sup> cDC1 and their CD103<sup>+</sup> counterparts in peripheral tissues) are specialized in antigen (Ag) cross-presentation, CD8<sup>+</sup> T cell activation and participate in antiviral immunity, cDC2 (SIRP $\alpha$ <sup>+</sup>CD11b<sup>+</sup>) are thought to have a predominant role in CD4<sup>+</sup> T cell priming and immune responses against extracellular pathogens. Importantly, cDC1 and cDC2 subpopulations can be further delineated into several smaller and less-well characterized tissue-specific subsets that exhibit diverse immune-modulatory functions. For example, splenic cDC2 consist of at least two predominant lineages, called cDC2A and cDC2B [31]. While cDC2A are characterized by high ESAM expression, cDC2B are ESAM<sup>lo</sup> and selectively express Clec12a [31–33]. ESAM<sup>hi</sup> cDC2A are efficient in MHC-II Ag-presentation and CD4<sup>+</sup> T cell activation, but poor in the production of pro-inflammatory cytokines, which is a characteristic of ESAM<sup>lo</sup> cDC2B [31–34].

Cell surface markers like ESAM, XCR1, and SIRP $\alpha$  can be used to analyze distinct splenic cDC populations by fluorescence-activated cell sorting (FACS). FACS is widely used for clinical applications and in life sciences due to its ability to isolate distinct cell populations with high purity including even rare subpopulations [35, 36]. Subsequent proteomic analysis of isolated cells by liquid chromatography–mass spectrometry (LC–MS) allows to further unravel cell-specific functionalities. MS is unparalleled in its ability to characterize and quantify proteins in an unbiased manner at the molecular level [37, 38]. By interfacing both techniques, FACS, and MS, it is possible to determine the cell-type-specific protein repertoire of distinct cellular subpopulations to decode their cell-type-specific proteomes and functionalities [39–41]. However, the proteomic analysis of a limited number of FACS-sorted cells can be very challenging. Often, substances used in the FACS sheath fluid including salts, polyethylene glycol (PEG), or other viscosity-altering molecules and/or components of FACS sample buffers (e.g. albumin, fetal calf serum (FCS)) can impair LC–MS downstream analysis. Hence, additional purification steps may be required prior to proteolytic digestion [40, 41] which can lead to sample loss and irreproducible results, particularly when handling low amounts of input material.

Here, we present a protocol combining FACS isolation of splenic cDC subsets with MS-based proteomic analysis. The protocol is optimized for sample types where cell numbers are limited and require 10,000–30,000 cells as input material, corresponding to between one and five micrograms of total protein, depending on DC subtype and maturation state. To keep purification steps at a minimum and avoid sample loss, pure PBS is used as sheath buffer, CD11c<sup>+</sup> splenocytes are washed with PBS prior to FACS analysis to remove FCS and are directly sorted into a sodium dodecyl sulfate (SDS)-containing lysis buffer. The workflow is compatible with single-pot, solid-phase-enhanced sample preparation (SP3) for proteomic analysis [41–43]. The presented protocol is devised and particularly attractive for the bulk characterization of cell-specific proteomes of rare, low abundant cell types.

### 4.2 Materials

#### 4.2.1 Reagents

All antibodies used in the present protocol are listed in Table 11.

All necessary reagents and chemicals are listed in Table 12.

##### 4.2.1.1 Buffers for single-cell preparation and FACS analysis.

**FCS:** Quickly thaw FCS at 37°C in a water bath. Once completely thawed, incubate for 60 minutes at 56°C in the water bath to destroy complement activity. Directly aliquot the warm FCS into 50 mL portions and store at –20°C. Avoid freeze-thaw cycles. Use aseptic techniques during the whole procedure.

**DNase I:** Dissolve Deoxyribonuclease I (DNase I) in PBS to reach a final concentration of 500 U/mL. Prepare aliquots, store dissolved DNase I at –20°C, and avoid freeze-thaw cycles. Use sterile solutions and aseptic techniques.

**Collagenase IV:** Dissolve Collagenase IV in PBS to reach a concentration of 10,000 U/mL. Prepare aliquots, store dissolved Collagenase IV at –20°C, and avoid freeze-thaw cycles. Use sterile solutions and aseptic techniques.

**Collagenase IV/DNase I Digestion mix:** Per mouse, prepare 1 mL of digestion solution containing 200 U/mL Collagenase IV and 0.5 U/mL DNase I in RPMI medium.

**FACS buffer:** Phosphate buffered saline solution (PBS) containing 3% FCS and 2 mM EDTA.

**PBS wash buffer.** Dissolve PBS powder (Roti®-PreMix PBS) according to manufacturer's instruction in LC–MS grade water. Can be stored at 4°C for up to 1 month and also be used for the preparation of the SDS lysis buffer.

**SDS lysis buffer (5 $\times$ ).** Dissolve 9.53 mg DDT and 25 mg SDS in 2.25 mL LC–MS grade water, add 250  $\mu$ L of PIC stock (50 $\times$ )

**Table 11.** Antibodies for FACS analysis

Monoclonal antibodies	Clone	Conjugate	Isotype	Manufacturer	cat. no.
Anti-CD11c	N418	FITC	Hamster IgG2	eBioscience	11.0114
Anti-CD172a (anti-SIRP $\alpha$ )	P84	PerCP-eFl710	Rat IgG1, $\kappa$	BD Biosciences	46-1721
Anti-ESAM	1G8/ESAM	PE	Rat IgG2a, $\kappa$	Biolegend	136203
Anti-I-A/I-E (anti-MHC-II)	M5/114.15.2	APC	Rat IgG2b, $\kappa$	Biolegend	107618
Anti-XCR1	ZET	BV510	Mouse IgG2b, $\kappa$	Biolegend	148218
Anti-CD16/32 (Anti-Fc $\gamma$ RIIB/III)	2.4G2	Purified	Rat IgG2a, $\lambda$	Biolegend	101302

to achieve a final concentration of 25 mM DTT, 10% (w/v) SDS and 5 $\times$  PIC concentration. Instead of water, PBS can be used as well. Prepare buffer freshly before use. *Note: Buffering capacity is provided by PBS from the sorting buffer.*

**4.2.1.2 Buffers and solutions for proteomic sample preparation (SP3 digest).**

**SP3 bead stock solution (20  $\mu$ g solids/ $\mu$ L).** Combine 20  $\mu$ L of Sera-Mag carboxylate-modified magnetic particles (hydrophobic,

**Table 12.** Reagents and chemicals used in the present protocol

Reagent/Chemical	Manufacturer	cat. no.
Single-cell preparation and FACS analysis		
ACK Lysing Buffer	Thermo Fisher Scientific Inc.	A1049201
Anti-CD11c Microbeads UltraPure, Hamster IgG2	Miltenyi Biotec	130-108-338
Collagenase Type IV	Worthington	LS0004186
cComplete Protease Inhibitor Cocktail (PIC)	Roche	1697498001
Deoxyribonuclease I (DNase I)	Roche	10104159001
1,4-Dithiothreitol 99%, p.a. (DTT)	Carl Roth	6908.1
Dulbecco's Phosphate Buffered Saline (PBS) w/o calcium and magnesium	Merck KGaA	D8537
Ethylenediaminetetraacetic acid (EDTA)	Merck KGaA	E5134-500G
Fetal Bovine Serum (FBS)	Gibco	10270-106
Fixable Viability Stain (L/D), FVS780-conjugated	BD Biosciences	565388
Roswell Park Memorial Institute (RPMI) 1640 Medium	Thermo Fisher Scientific Inc.	11530586
Roti $\textcircled{R}$ -PreMix PBS	Carl Roth	0890.1
Sodium dodecyl sulfate (SDS)	AppliChem	A7249-0250
Water Rotisolv $\textcircled{R}$ , Ultra LC-MS	Carl Roth	HN43.1
Proteomic sample preparation (SP3 digest)		
Absolute ethanol	Merck KGaA	34852-M
Acetic acid	Carl Roth	3738.2
Acetonitrile (ACN), Rotisolv $\textcircled{R}$ , Ultra LC-MS	Carl Roth	HN40.1
Ammonium bicarbonate	Carl Roth	T871.1
1,4-Dithiothreitol 99%, p.a. (DTT)	Carl Roth	6908.1
Dimethylsulfoxide (DMSO)	Carl Roth	HN47.1
Formic acid (FA), ROTIPURAN $\textcircled{R}$ , LC-MS Grade	Carl Roth	1EHK.1
Iodoacetamide BioUltra (IAA)	Merck KGaA	I1149-25G
Sera-Mag $\text{TM}$ Magnetic carboxylate modified particles (hydrophilic)	Merck	GE24152105050250
Sera-Mag $\text{TM}$ magnetic carboxylate modified particles (hydrophobic)	Merck	GE44152105050250
Trypsin Gold, MS Grade	Promega	V5280
Water Rotisolv $\textcircled{R}$ , Ultra LC-MS	Carl Roth	HN43.1
LC-MS analysis		
Acetonitrile (ACN), Rotisolv $\textcircled{R}$ , Ultra LC-MS	Carl Roth	HN40.1
Formic acid (FA), ROTIPURAN $\textcircled{R}$ , LC-MS Grade	Carl Roth	1EHK.1
Isopropanol (2-Propanol) Rotisolv $\textcircled{R}$ , Ultra LC-MS	Carl Roth	0733.1
Sodium hydroxide	Carl Roth	6771.2
Water Rotisolv $\textcircled{R}$ , Ultra LC-MS	Carl Roth	HN43.1

provided at a concentration of 50  $\mu\text{g}/\mu\text{L}$  by the vendor) and 20  $\mu\text{L}$  of Sera-Mag Speed-Bead carboxylate-modified magnetic particles (hydrophilic, 50  $\mu\text{g}/\mu\text{L}$ ) in a 1.5 mL-tube. Add 160  $\mu\text{L}$  of water and mix. Place the tube with beads on a magnetic rack and remove supernatant after beads have settled. Off the rack, add 200  $\mu\text{L}$  of water and mix. Repeat wash steps two further times. Recover beads in 100  $\mu\text{L}$  of water. SP3 bead stock can be stored at 4°C for up to 1 month.

**1.4-Dithiothreitol (DTT) stock solution (200 mM).** Weigh 3–5 mg of DTT directly into a fresh 1.5-mL tube and dissolve in water/or PBS to a final concentration of 200 mM. As DDT is oxygen-sensitive DDT stock solutions and DTT-containing buffers should be prepared freshly before use.

**Iodoacetamide (IAA) stock solution (400 mM).** Weigh 10–20 mg of IAA directly into a fresh 1.5-mL tube and dissolve in water/or PBS to a final concentration of 400 mM. As IAA is light-sensitive IAA stock solutions and IAA-containing buffers should be prepared freshly before use and kept in the dark.

**70% (v/v) ethanol (EtOH).** Mix 70 mL of absolute EtOH with 30 mL of water.

**Ammonium bicarbonate buffer (50 mM).** Weigh 40 mg of ammonium bicarbonate and transfer to a 15 mL-polypropylene tube. Dissolve in 10 mL of water to a final concentration of 50 mM. Prepare freshly prior to use.

**Trypsin stock solution (1  $\mu\text{g}/\mu\text{L}$ ).** Dissolve lyophilized trypsin in 50 mM acetic acid prepared in water to a final concentration of 1  $\mu\text{g}/\mu\text{L}$ . Store 5  $\mu\text{L}$ -aliquots at  $-80^\circ\text{C}$ .

**Trypsin digestion buffer.** Dilute trypsin stock solution in ammonium bicarbonate buffer. For proteolytic digestion, trypsin-to-protein ratio should be approx. 1:25 (w/w) and the final working volume 5  $\mu\text{L}$ . Prepare freshly before use.

**Peptide elution solution, 2% (v/v) DMSO.** Mix 98  $\mu\text{L}$  of water with 2  $\mu\text{L}$  of DMSO. Prepare freshly before use.

#### 4.2.1.3 Buffers and solutions for LC-MS analysis.

**Solvent A.** 0.1% (v/v) FA in water. Degas solvent, either by sparging with helium or sonication for at least 15 min.

**Solvent B.** 0.1% (v/v) FA in ACN. Degas solvent, either by sparging with helium or sonication for at least 15 min.

**Needle and fluidics wash.** Wash solution A: 1% (v/v) FA in water. Wash solution B: 0.1% (v/v) FA in ACN. Degas solvents, either by sparging with helium or sonication for at least 15 min.

**Transport liquid.** 0.1% FA (v/v) in water. Prepare freshly (i.e. once a week).

**Calibration solution, 10 mM sodium formate.** To prepare a 10 mM sodium formate calibration solution, mix 12.5 mL water, 12.5 mL isopropanol, 50  $\mu\text{L}$  formic acid, and 250  $\mu\text{L}$  sodium hydroxide.

Equipment required for the present protocol is listed in Table 13.

### 4.3 Step-by-step sample preparation

#### 4.3.1 Single-cell preparation for FACS analysis

1. Euthanize mice using  $\text{CO}_2$ .
2. Dissect out the spleen.
3. Cut tissue in grain size pieces and incubate in 1 mL digestion mix while shaking at  $37^\circ\text{C}$  for 30 min.
4. Add EDTA to a 10 mM final concentration and incubate the cell suspension for 5 min at  $4^\circ\text{C}$ .
5. Add cell suspension onto a 40  $\mu\text{m}$  cell strainer (placed on a 50 mL tube).
6. Wash cell strainer by adding 10 mL FACS buffer.
7. Centrifuge cells for 5 min at  $400 \times g$  at  $4^\circ\text{C}$ . Aspirate supernatant completely.
8. Resuspend cell pellet in 1 mL ACK lysis buffer to lyse red blood cells. Incubate at room temperature for 5 min.
9. Add 10 mL of FACS buffer.
10. Centrifuge cells for 5 min at  $400 \times g$  at  $4^\circ\text{C}$ . Aspirate supernatant completely.
11. Resuspend cell pellet in 400  $\mu\text{L}$  FACS buffer.
12. Commercially available, purified rat anti-mouse anti-CD16/32 is employed to block the Fc-gamma RIIB/III receptors, thereby avoiding unspecific recognition of staining antibodies by cell-bound Fc-gamma receptors. For this, calculate the amount of Fc-block and prepare in 25  $\mu\text{L}$  FACS buffer per well.
13. Apply the 25  $\mu\text{L}$  Fc solution onto cells, mix well and incubate for 5 min at  $4^\circ\text{C}$ .
14. To isolate CD11c<sup>+</sup> dendritic cells from the single-cell suspension, incubate cells from one spleen with 100  $\mu\text{L}$  anti-mouse CD11c MACS MicroBeads UltraPure. Perform MACS purification and isolation according to the manufacturer's protocol.
15. Elute the CD11c<sup>+</sup> cDC from the LS MACS column by applying 5 mL of FACS buffer to the column, followed by firmly pushing the plunger into the column.
16. Count isolated cells and continue with section 4.3.2.

#### 4.3.2 Flow cytometric purification, collection and lysis of splenic cDC subsets

1. Prior to FACS analysis, aliquot SDS lysis buffer (5 $\times$ ) in fresh 1.5 mL protein LoBind Eppendorf tubes. Note: Depending on the nozzle and sorting mode, calculate the volumes of 10% (w/v) SDS lysis buffer (5 $\times$ ) required to achieve a final concentration of 2% (w/v) SDS, 5 mM DTT, and 1 $\times$  PIC

**Table 13.** Summary of required equipment

Equipment	Manufacturer	cat. no.
Single-cell preparation and FACS analysis		
2 mL microcentrifuge tubes	Sarstedt AG	72.695.200
15 mL tubes	Greiner bio-one	188271 or equivalent
50 mL tubes	Greiner bio-one	227261 or equivalent
BD FACSAria I	BD Biosciences	
Centrifuge “Z 446 K”	Hermle LaborTechnik	
FACS tube	Corning	352008
Falcon® 40µm cell strainer	Corning	352340
MACS LS Columns	Miltenyi Biotec	130-042-401
Neubauer chamber 0.100 mm; 0.0025 mm <sup>2</sup>	Superior Marienfeld	
PipetBoy	Fisher Scientific	11701258 or equivalent
PipetMan (P10-P1000)	Gilson	
Pipette tips	Brand	
Protein LoBind 1.5 mL Eppendorf tubes	Eppendorf	0030108116
QuadroMACS Separator	Miltenyi Biotec	
Serological pipettes (1-25 mL)	Gilson	
Proteomic sample preparation (SP3 digest)		
Bioruptor Plus device	Diagenode	B01020001 or equivalent
Benchtop centrifuge holding 1.5-mL tubes (up to 21,000 x g)	Thermo Scientific	10406173 or equivalent
Magnetic rack(s) holding 1.5-mL microfuge tubes	Cell Signaling	7017S or equivalent
Protein LoBind 1.5 mL Eppendorf tubes	Eppendorf	0030108116
Protein low binding pipet tips (Axygen®Maxymum Recovery®Universal Fit Pipet Tips)	Corning	TF-20-L-R-S, TR-222-C-L-R, TF-1000-L-R-S
ThermoMixer equipped with a 1.5-mL tube holder	Grant Instruments	97011-930 or equivalent
LC-MS analysis		
Aurora Series UHPLC column + CaptiveSpray insert	Ionopticks	AUR2-25075C18A-CSI
CaptiveSpray nanoBooster Source	Bruker Corporation	1820410 or equivalent
Column toaster	Bruker Corporation	1862996 or equivalent
Nalgene™ syringe filter	Thermo Scientific	722-2000
Syringe filter, PTFE, with active charcoal reservoir	Sartorius	17840——Q
nanoElute® LC system	Bruker Corporation	1832734 or equivalent
timsTOF Pro (2)	Bruker Corporation	1900000 or equivalent
Total recovery glass vials	Waters Corporation	600000750cv

after the cell sorting; e.g. 22.5 µL of 10% (w/v) SDS lysis (5×) buffer are needed for the lysis of 30,000 cells when using a 100 µm nozzle and 4-way purity mode. According to the manufacturer droplet size per cell corresponds to 3 nL when using these settings resulting in a total “sort” volume of 90 µL.

- Calculate and prepare the antibody master mix. *Note: Don't forget to include controls and optional extra staining for instrument setup.*
- Centrifuge cells for 5 min at 400 x g at 4°C. Aspirate supernatant completely.
- Apply 200 µL antibody master mix onto cells, mix well by pipetting up and down and incubate for 30 min at 4°C in the dark (fridge).
- Fill tubes with 5 mL FACS buffer.
- Centrifuge cells for 5 min at 400 x g at 4°C. Aspirate supernatant completely.

- Wash cells by adding 2 mL PBS prepared with LC-MS grade water, resuspend the cells and spin down for 5 min at 400 x g at 4°C. Aspirate supernatant completely. Repeat this washing step.
- Resuspend cells at a concentration of  $1 \times 10^7$  cells/mL in PBS (prepared with LC-MS grade water). *Note: Do not use volumes below 500 µL for resuspension of cells.*
- Sort cDC subsets by gating on high expression of CD11c and MHCII and the absence or presence of SIRPα, ESAM, and XCR1. Sort 30,000 cells directly into a protein LoBind Eppendorf tube containing  $5 \times$  SDS-lysis buffer (see 3.2. step 1). To sort cDC subpopulations, we used a nozzle size of 100 µm, and “4-way purity” sort mode, which required 22.5 µL of  $5 \times$  SDS-lysis buffer.
- Incubate samples directly after FACS isolation at 95°C for 5 min to promote cell disruption and protein solubilization.
- Cool down samples for 5 min on ice.



12. Place samples into the Bioruptor device and sonicate at 4°C at maximum intensity applying in total 15 cycles (with 30 s on/off intervals each) to further promote cell lysis and to shear chromatin.
13. Process samples by single-pot solid-phase-enhanced sample preparation (SP3, see section 3.4.). *Pause point: cellular lysates can be also stored at -80°C until further processing.*

#### 4.3.3 Proteolytic digestion using single-pot solid-phase-enhanced sample preparation (SP3)

1. Reduce disulfide bonds by adding DTT stock solution to the samples resulting in a final concentration of 10 mM DTT (e.g. 0.5  $\mu$ L of DTT stock solution per 10  $\mu$ L of lysate). Incubate samples at 45°C for 30 min.
2. To alkylate free cysteine residues, add IAA stock solution to achieve a final concentration of 40 mM (e.g. 1  $\mu$ L per 10  $\mu$ L of lysate). Incubate samples in the dark for 30 min at room temperature.
3. To quench the alkylation reaction, add 0.75  $\mu$ L of DTT solution per 10  $\mu$ L of lysate resulting in a final concentration of 15 mM DTT.
4. Shortly vortex the SP3 bead stock solution. Add 2  $\mu$ L of the bead stock to each sample. Shortly vortex-mix the suspension and avoid excessive pipetting to prevent sample loss. *Note: If the sample volume exceeds 50  $\mu$ L, adjust the amount of beads that are added to achieve a concentration of at least 0.5  $\mu$ g/ $\mu$ L of SP3 beads in the final bead-sample suspension.*
5. Add ACN to a final concentration of 70% (v/v) to induce aggregation and binding of proteins to the SP3 beads. Vortex-mix the samples immediately.
6. Incubate the samples for 20 min at room temperature. Briefly vortex-mix the samples during the first 10 min as soon as sedimentation of the protein-bead aggregates is observed. Afterward, allow the beads to settle.
7. Place the samples into a magnetic rack to capture the beads at the tube wall. Wait at least 2 min until all beads are migrated to the tube wall. Remove and discard the supernatant.
8. Rinse the pelleted SP3 beads on the magnetic rack by adding and carefully pipetting up and down 200  $\mu$ L of 70% (v/v) EtOH. Remove and discard the EtOH-containing supernatant. Repeat this step a second time with 70% (v/v) EtOH and once with ACN. Transfer the tubes with opened lid from the magnetic rack and let residual ACN evaporate.
9. Add 5  $\mu$ L of trypsin digestion buffer and resuspend beads. Incubate the samples at 37°C overnight to promote tryptic digestion of proteins. *Note: We typically use a trypsin-to-sample ratio of 1:25 (w/w). To estimate the protein content in dendritic cells, we used DC 2.4 cells as reference, which contain around 80–90 pg of protein per cell corresponding to approx. 2.4–2.7  $\mu$ g per 30,000 cells. Hence, 5  $\mu$ L of digestion buffer should contain around 108 ng of trypsin.*
10. After tryptic digestion, resuspend the beads by shortly vortexing the tubes. To purify the peptide mixture and promote

peptide binding to the beads, add ACN to a final concentration of 95% (v/v). Immediately vortex-mix the samples.

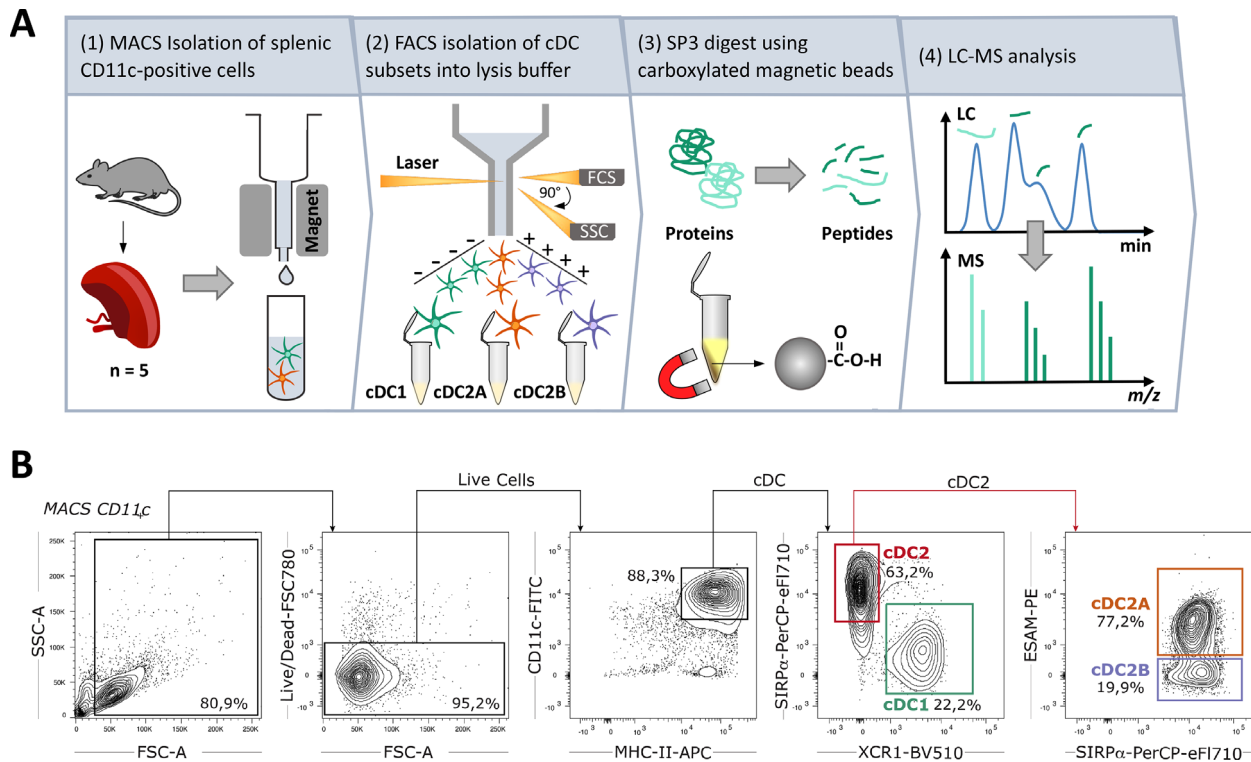
11. Incubate the samples for 20 min at room temperature. Briefly vortex-mix the samples during the first 10 min as soon as sedimentation of the peptide-bead aggregates is observed. Afterward, allow the beads to settle.
12. Place the samples into a magnetic rack and capture the beads at the tube wall. Wait at least 2 min until all beads are migrated to the tube wall. Remove and discard the supernatant. *Note: To increase the peptide recovery, the supernatant can be incubated a second time with SP3 beads [41] (see also section 5). Continue with step 11.*
13. Rinse the pelleted SP3 beads on the magnetic rack by adding and carefully pipetting up and down 200  $\mu$ L of ACN. Remove and discard the supernatant. Transfer the tubes with opened lid from the magnetic rack and let residual ACN evaporate.
14. Reconstitute the beads in 10  $\mu$ L of 2% (v/v) DMSO to elute purified peptides from the SP3 beads. Incubate the samples in an ultrasonic bath for 1 min to improve peptide recovery. *Note: If peptides were recovered a second time with SP3 beads (see “Note” step 12), combine both SP3 beads in a total of 10  $\mu$ L 2% (v/v) DMSO.*
15. Centrifuge the tubes for 2 min at 12,500 x g and place them onto the magnetic rack. Recover the supernatant containing tryptic peptides after all beads have settled on the tube wall. Make sure not to recover any beads.
16. To proceed with LC-MS analysis, mix samples with diluted FA to a final concentration of 0.1% (v/v) FA. If you opt for a final sample volume of 20  $\mu$ L, for example, add 10  $\mu$ L of 0.2% (v/v) FA to the sample.

#### 4.3.4 Liquid chromatography-mass spectrometry (LC-MS)

*Note:* In the present study, samples were analyzed on a timsTOF Pro 2 mass spectrometer coupled to a nanoElute LC system (Bruker Corporation) using ion mobility-enhanced data-independent acquisition (DIA-PASEF) as described by Meier et al. [44] However, mass spectrometric analysis of the samples can be conducted on any high-resolution, state-of-the-art LC-MS instrument platform.

1. Transfer samples into glass vials and place them into the autosampler of your LC system. In the present study, we used a nanoElute LC platform for the separation of tryptic peptides.
2. Mount the CaptiveSpray source on the timsTOF Pro 2 mass spectrometer.
3. Install a system comprising reversed-phase C18 column (e.g. Aurora UHPLC emitter column, 25 cm x 75  $\mu$ m 1.6  $\mu$ m, IonOpticks).
4. Perform mass and tims calibration according to the manufacturer's protocol.
5. To setup your chromatographic method, choose “one-column separation” mode in the chromatographic method editor, set the column temperature to 50°C and choose default settings





**Figure 7. Proteomic workflow and gating strategy for the analysis of cDC subsets.** A) Graphical illustration of the presented proteomic protocol combining FACS isolation and LC-MS downstream analysis of splenic cDC subsets. (B) Flow cytometry gating strategy for the isolation of splenic cDC subsets. Representative cytometry plots of the gating strategy used for sorting  $XCR1^+ SIRP\alpha^-$  cDC1 and  $XCR1^- SIRP\alpha^+$  cDC2 from the mouse spleen. CD11c MACS-enriched splenocytes from 10 to 16-week-old mice were stained with the indicated antibodies, and subsequently, cDC were defined as viable gated  $CD11c^+ MHC-II^+$  cells. cDC subsets were based on XCR1 and  $SIRP\alpha$ , where the expression or absence of ESAM further defined cDC2 as cDC2A or cDC2B populations, respectively [31, 33, 45].

for separation column equilibration and sample loading *Note:* We set the column equilibration volume to 5.0 instead of 4.0. Additionally, we lowered equilibration and sample loading pressure to 600 bar to increase column life time.

- Program the following gradient for peptide separation in the method editor: Increase solvent B from 2% (v/v) to 25% (v/v) in 34.5 min, then to 37% (v/v) over 3.8 min, followed by ramping up B% to 95% (v/v) over another 3.8 min. Wash column for 5 min with 95% (v/v) B. Set the flow rate to 400 nL/min. *Note:* We would recommend to adjust gradient according to column and sample type.
- Analyze eluting peptides in positive mode ESI-MS on a timsTOF Pro 2 instrument. We recommend to analyze the samples using parallel accumulation serial fragmentation (PASEF)-enhanced data-independent acquisition mode (DIA) as described in detail by Meier et al. [44] Operate the dual TIMS (trapped ion mobility spectrometer) at a fixed duty cycle close to 100% using equal accumulation and ramp times of 100 ms each spanning a mobility range from  $1/KO = 0.6 \text{ Vs cm}^{-2}$  to  $1.6 \text{ Vs cm}^{-2}$ . Define  $36 \times 25$  Th isolation windows from  $m/z$  300 to 1,165 (i.e. fifteen diaPASEF scans per acquisition cycle). Ramp the collision energy linearly as a function of the mobility from 59 eV at  $1/KO = 1.3 \text{ Vs cm}^{-2}$  to 20 eV at  $1/KO = 0.85 \text{ Vs cm}^{-2}$ .

#### 4.4 Data analysis

Here, we describe a detailed protocol for the MS-based proteomic characterization of mouse splenic cDC1 and cDC2 subsets isolated by FACS (Fig. 7A). The detailed gating strategy for the selection of cDC1 ( $CD11c^+ MHC-II^+ XCR1^+ SIRP\alpha^-$ ), cDC2A ( $CD11c^+ MHC-II^+ SIRP\alpha^+ ESAM^+ XCR1^-$ ) as well as cDC2B ( $CD11c^+ MHC-II^+ SIRP\alpha^+ ESAM^- XCR1^-$ ) cells is summarized in Fig. 7B. Cells were directly sorted into SDS-lysis buffer and processed for LC-MS analysis by SP3 to minimize sample loss. The exemplary dataset provided with this protocol contains five biological replicates of each cDC subpopulation. LC-MS raw data processing, peptide identification, and label-free quantification (LFQ) analysis of proteins were performed using DIA-NN (v1.8) [46]. To this end, samples were processed using library-free mode with standard parameters including some slight modifications as summarized in Table 14. The required FASTA protein database containing 17,068 reviewed protein entries of mouse and 172 common contaminant proteins was obtained on April 16, 2021, from uniprot.org.

To identify differentially regulated proteins in the different cDC subsets, further statistical analysis was conducted using the protein level LFQ values reported by DIA-NN. A two-sided t-test assuming equal variances was performed considering only proteins that had been identified in at least three biological

**Table 14.** Settings used for data processing in DIA-NN

Parameter	Setting used
Precursor ion generation	
FASTA digest for library-free search/library generation	library-free search selected
Deep learning-based spectra, RTs, and IMs prediction	selected
Protease	Trypsin
Missed cleavages	default setting used
Modifications	default setting used
Peptide length range	default setting used
Precursor charge range	changed to 1–6
Precursor <i>m/z</i> range	default setting used
Fragment ion <i>m/z</i> range	default setting used
Output	
Additional options	Command “relaxed-prot-inf” applied for homology filtering
Algorithm	
Mass accuracy	default setting used
Unrelated runs	default setting used
MS1 accuracy	default setting used
Use isotopologues	default setting used
Scan window	default setting used
MBR	MBR selected
Neural network classifier	default setting used
Protein inference	set to “genes species-specific”
Quantification strategy	default setting used
Cross-run normalisation	default setting used
Library generation	default setting used
Speed and RAM usage	default setting used

replicates and were identified by at least two peptides. The resulting *p*-values were corrected for multiple testing using the Benjamini-Hochberg method. Only proteins with an adjusted *p*-value below 0.01 and a fold change of at least 1.4 as compared to the other groups were included in the list of cDC subset-specific proteins (Fig. 8). The statistical programming language R including the pheatmap [47], imputeLCMD [48] and pcaMethods [49] packages was applied to conduct further analyses and to visualize results (see Fig. 8A and 8B). Functional annotation analysis was performed using the DAVID knowledgebase (Database for Annotation, Visualization and Integrated Discovery, version 6.8) (<https://david.ncifcrf.gov/>) [50]. Venn diagram data were calculated using the Venny web application (<http://bioinfo.gp.cnb.csic.es/tools/venny/index.html>).

In total, 4,747 proteins were identified across the whole dataset revealing distinct cDC subset-specific differences that undermine the power of integrated FACS and LC-MS analysis (Fig. 8). Besides the cell surface markers used for FACS-sorting (Fig. 8D, left panel) a multitude of additional cell type-specific proteins could be identified by the proteomic analysis, including different transmembrane proteins and cell-surface receptors,

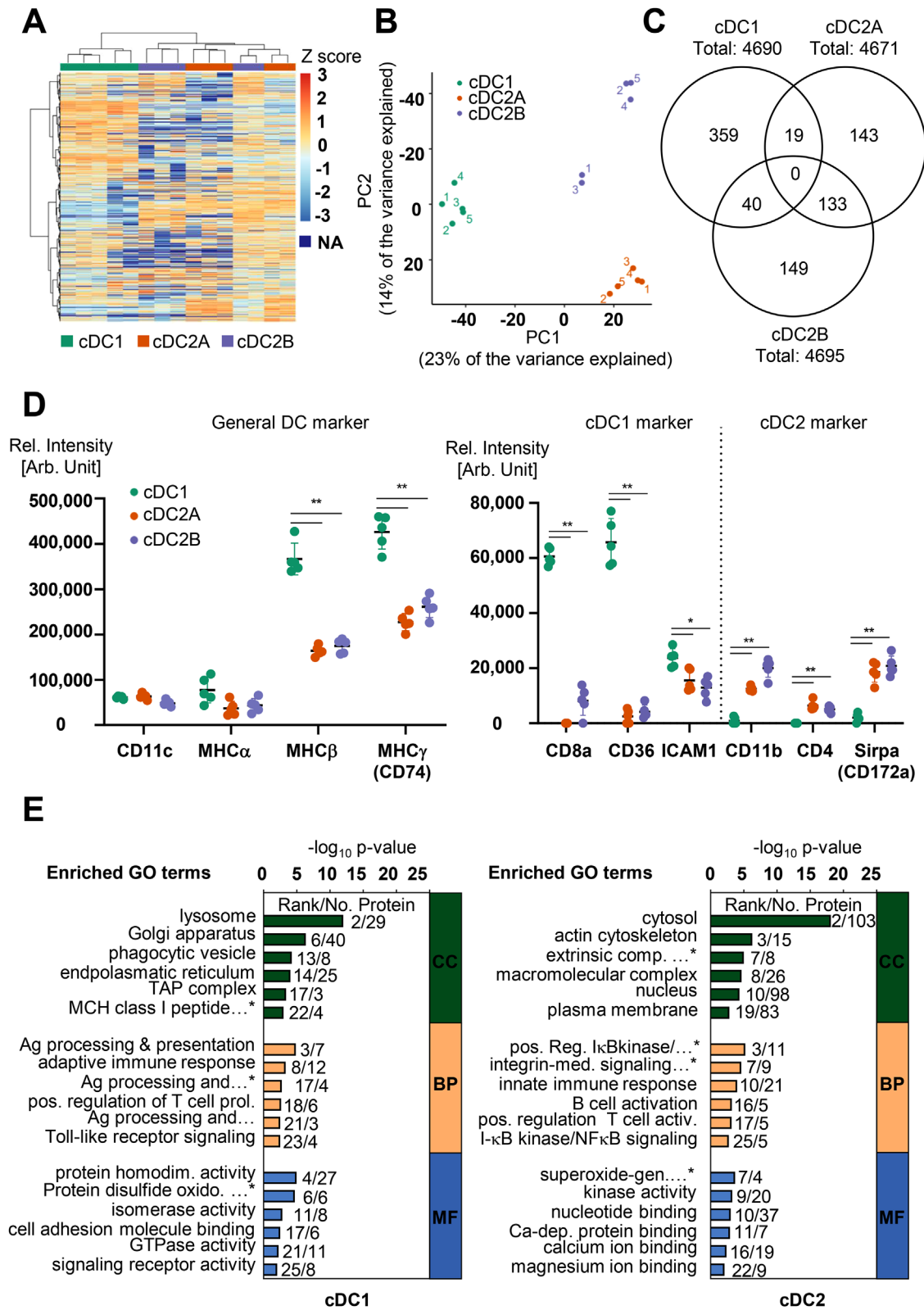
cytosolic proteins as well as transcription factors (see Table 15). Gene ontology analysis revealed an enrichment of proteins associated with antigen processing and presentation via MHC class I in cDC1 cells, whereas proteins identified in cDC2 cells are mainly involved in proinflammatory processes (e.g. regulation of interferon-gamma production, regulation of TNF production) (Fig. 8E).

#### 4.5 Pitfalls

Different protocol steps markedly influence the overall performance of the workflow and to achieve good, satisfactory results, the following pitfalls should be avoided.

One major aspect which is critical for a successful LC-MS experiment is the sample quality. To avoid any problems arising from low-quality reagents, all (sample) buffers should be prepared using either HPLC/LC-MS grade or ultrapure water (prepared by purifying deionized water to attain a conductivity of 18 MΩ cm<sup>-1</sup> at 25°C) as well as analytical grade reagents. DDT- and IAA-containing buffers should always be prepared freshly before use as DDT and IAA are sensitive to oxygen and light, respectively. Hence, IAA-containing buffers should be kept in the dark. Moreover, particularly during FACS analysis and SP3 sample processing, make sure to work in a clean environment and to always wear appropriate gear (i.e. laboratory coat and nitrile gloves) to minimize sample contamination with keratins derived from dead skin (or hair). For the preparation of LC mobile phases and wash solutions, exclusively use LC-MS grade solvents and acids as low-quality solvents can markedly impair and reduce the performance of LC-MS experiments. Additionally, make sure that LC-MS solvents are degassed properly prior to use (see also section 4.2.1.3).

Each sample transfer and processing step can lead to protein and peptide loss, particularly when working with a limited amount of input material. The SP3 procedure already markedly minimizes processing and transfer steps as compared to other proteomic protocols [41–43]. Nevertheless, to further reduce loss during sample preparation, it is crucial to use protein low-binding tubes and pipette tips for sample processing and the collection of FACS-sorted cells (see also equipment setup – section 4.2.2., Table 13). In the present protocol, cellular lysates are generated by directly sorting cDC subsets into an SDS-based lysis buffer (5× concentrated). Hence, to achieve optimal lysis conditions, i.e. to ensure that the lysis buffer is not diluted below 1–2% (w/v) SDS and lysis will be efficient, it is critical to estimate the total sort volume prior to the experiment. The total sort volume is dependent on the target number of cells, the size of the nozzle as well as the sorting mode and should be calculated taking these parameters into account (see also example calculation in section 4.3.2, step 1). We highly recommend to perform a pilot experiment using the chosen FACS settings. Check if calculated (theoretical) volumes match experimental ones. This way you can ensure that the proper amount of SDS-lysis buffer (5×) required for efficient lysis will be applied in the main experiment. Once cells are collected by FACS, make sure to directly promote cell lysis by boiling samples



**Figure 8.** LC-MS analysis of FACS-purified cDCs reveals subset-specific protein expression patterns. Label-free quantitative proteome analysis of FACS-purified splenic cDC1, cDC2A, and cDC2B cells derived from C57BL/6J mice (n = 5). (A) Heatmap of all quantified proteins in the dataset. For cluster analysis and heatmap visualization, label-free quantification values were log<sub>2</sub>-transformed and Z-score normalized for each protein. (B) Principal component analysis of the transformed dataset identified three distinct clusters along with the first and second principal component corresponding to the different cDC subsets. (C) Venn Diagram of differentially expressed proteins in cDC1, cDC2A, and cDC2B (Benjamini-Hochberg

for 5 min (section 4.3.2, step 10) to avoid uncontrolled lysis and protein degradation when leaving samples at room temperature for a prolonged time interval.

Another critical step is the initial protein capture to the SP3 beads. Here, several points need to be taken into consideration. One is the overall sample volume after sorting (including lysis buffer), which at best should not exceed 100–120  $\mu\text{L}$  for the initial protein binding step in SP3. Hence, we suggest to sort with a nozzle and sorting mode that creates small droplets per cell. In our hands, working volumes between 30–100  $\mu\text{L}$  and 1–10  $\mu\text{g}$  of total protein achieved best results for SP3 sample processing [41]. Based on the bead binding capacity the ideal SP3 bead-to-protein ratio is 10:1 (w/w) [43] at a final bead concentration of at least 0.5  $\mu\text{g}/\mu\text{L}$  [42, 43]. However, when analyzing limited numbers of FACS-sorted cells these conditions are hard to meet. Typically, protein amounts do not exceed 3  $\mu\text{g}$  of protein upon lysis of 10,000–30,000 primary immune cells and could even range below 1  $\mu\text{g}$ . Hence, bead quantity should be adapted to the sample volume as suggested by Hughes *et al.* [42] who recommend a working concentration of 0.5  $\mu\text{g}$  beads per 1  $\mu\text{L}$  of processing volume in an SP3 sample where protein input material is low. For example, to 100  $\mu\text{L}$  of a working (sample) volume 2.5  $\mu\text{L}$  of bead stock should be added, resulting in a final bead concentration of 0.5  $\mu\text{g}/\mu\text{L}$ . Besides the amount of beads, we found that the pH is a critical parameter during the initial protein-bead binding step. In contrast to the original SP3 protocol where initial protein capture is performed at acid pH [43], cDC lysates are incubated with the SP3 beads at a neutral pH in the present protocol. As acidic conditions can impede protein binding to the carboxylate-coated beads and lead to increased variability [41, 51], we recommend to work at neutral pH. In addition to the protein, intact chromatin or RNA can also bind to the magnetic carboxylate-coated SP3 beads and interfere with the SP3 protocol. In case of the present workflow, we recommend DNA and RNA shearing by means of a suitable ultrasonication device to further aid cell disruption and protein extraction. Using a Bioruptor device with the settings described in section 4.3.2, step 12, is typically sufficient to shear DNA and RNA. Alternatively, RNA and DNA can also be degraded enzymatically, e.g. by benzonase treatment of the cDC lysates.

To increase sample recovery, i.e. to make sure that no peptides are lost after the overnight tryptic digest, optionally the SP3 peptide purification step can be repeated with the supernatant of the initial peptide-binding step (see note, Section 4.3.3, step 12). To do so, do not discard the supernatant during the first step of peptide purification. Instead, collect the supernatant, add 2  $\mu\text{L}$  of

carboxylate-functionalized bead mix, and repeat the ACN wash and recovery steps. Peptides derived from the initial binding step as well as from the second purification step can then be pooled prior to MS analysis [41].

A multitude of additional helpful tips and tricks as well as common pitfalls regarding the SP3 sample processing itself are discussed in depth by Hughes *et al.* [42], which we would highly recommend for further reading.

#### 4.6 Top tricks

To improve the performance of the present protocol, we recommend to estimate the protein content in the samples as this has a major impact on several protocol steps. One example would be the LC-MS measurement itself. There is a “sweet spot” for each LC-MS setup regarding the optimal sample on-column load that will yield the best results in terms of identified peptides and proteins as well as quantitative reproducibility. Another example is the proteolytic digest. Knowing the protein content of your sample can help estimate the trypsin amount required for efficient proteolytic digestion. Moreover, too high amounts of trypsin, for example, can result in abundant trypsin autolysis products that might dominate and impair LC-MS analysis and results.

Typically, when sample material is not limited, the total protein content of cellular lysates can be determined by colorimetric protein assays, such as the Pierce 660 nm protein assay (Thermo Fisher Scientific). However, when the amount of starting material is limited, as is the case for certain cDC subpopulations, performing a protein assay likely consumes significant amounts of sample prior to LC-MS downstream analysis. In order to estimate the protein content of the actual samples of interest, it is recommended to perform preliminary experiments either with the cell type of interest itself or with similar but less rare sample types such as cell lines (see also note, Section 4.3.3, step 9).

To determine the optimal on-column load for LC-MS experiments, it is also possible to estimate the sample yield at the peptide level performing either fluorometric or absorbance-based assays, such as the Pierce Quantitative Colorimetric Peptide Assay (Thermo Fisher Scientific) or measurements at 205 nm /280 nm. Alternatively, a pre-measurement of samples can be conducted by LC-MS and used to calculate optimal injection amounts for the main analysis. Besides the sample on-column load, there are a multitude of additional factors that influence the performance of LC-MS measurements, which are beyond the scope of the present protocol. These include, for example, different LC settings (i.e.

← correction  $p < 0.01$ ,  $\log_2$ -transformed fold change compared to other cDC subsets  $> 0.5$ ) reveals cell type-specific proteins for each cDC subpopulation. Total number of proteins identified in each subset is indicated. (D) Relative LFQ intensities of selected cDC markers. In the left panel cDC markers used for FACS analysis are displayed. In the right panel selected markers for cDC1 and cDC2 subsets are depicted. (E) Gene Ontology (GO) enrichment analysis of proteins that are significantly associated with either cDC1 or cDC2 subsets (Benjamini–Hochberg correction  $p < 0.01$ ,  $\log_2$ -transformed fold change compared to the other cDC subset  $> 1$ ). Full names of GO terms marked with asterisk (\*): MHC class I peptide loading complex, antigen processing and presentation of exogenous peptide antigen via MHC class II, antigen processing and presentation of endogenous peptide antigen via MHC class I, protein disulfide oxidoreductase activity, extrinsic component of cytoplasmatic side of plasma membrane, positive regulation of I-kappaB kinase/NK-kappaB signaling, integrin-mediated signaling pathway, superoxide-generating NADPH oxidase activity.



**Table 15.** Overview of mouse splenic cDC phenotypes and cell-specific markers

cDC subpopulation	Surface marker used for FACS analysis	Markers identified by proteome analysis	
		Shared cDC markers (no differential expression)	Celltype-specific markers (differentially expressed)
cDC1	CD11c, MHC-II, XCR1	ITGAX (CD11c), CD45, MHC $\alpha$	BTLA (CD317), CADM1 (NECL2), CD8 $\alpha$ , CLEC9a (CD370), IRF8, ITGAE (CD103), LY75 (DEC205, CD205), TLR3, Tap1, Tap2, MHC $\beta$ , MHC $\gamma$ , CD36, ICAM1
cDC2A	CD11c, MHC-II, Sirp $\alpha$ , ESAM	ITGAX (CD11c), CD45, MHC $\alpha$	FCER1, IRF4, ITGAM (CD11b), Sirp $\alpha$ (CD172a), TLR7 <sup>1</sup> , CD4, RelB, RUNX3, IKZF1 (IKAROS), Zeb2 <sup>1</sup>
cDC2B	CD11c, MHC-II, Sirp $\alpha$	ITGAX (CD11c), CD45, MHC $\alpha$	CLEC10a (CD301), IRF4, ITGAM (CD11b), Sirp $\alpha$ (CD172a), TLR7 <sup>1</sup> , CD4, RelB, RUNX3, IKZF1 (IKAROS), Zeb2 <sup>1</sup> , Lyz2

<sup>1</sup> ns: close to detection limit – hence t-test not significant, but absent in all cDC1 runs

LC-gradient, flow rate, column type, etc) as well as MS instrument parameters and MS acquisition modes that might also be instrument dependent. As the samples generated by the present proteomic protocol can be analyzed on any state-of-the-art instrument LC-MS platform, we highly recommend checking the respective literature or vendor recommendations. Moreover, we advise to use data-independent acquisition methods for label-free quantitative proteomic analysis [52–55], as the resulting datasets display a significantly enhanced reproducibility and fewer missing values compared to data-dependent acquisition workflows.

## 4.7 Summary table

Table 15 lists cDC subpopulation-specific markers used for FACS analysis as well as cell-type-specific markers identified by LC-MS analysis.

## 5 Gelatin degradation assay to address podosome formation and function of mouse DC

### 5.1 Introduction

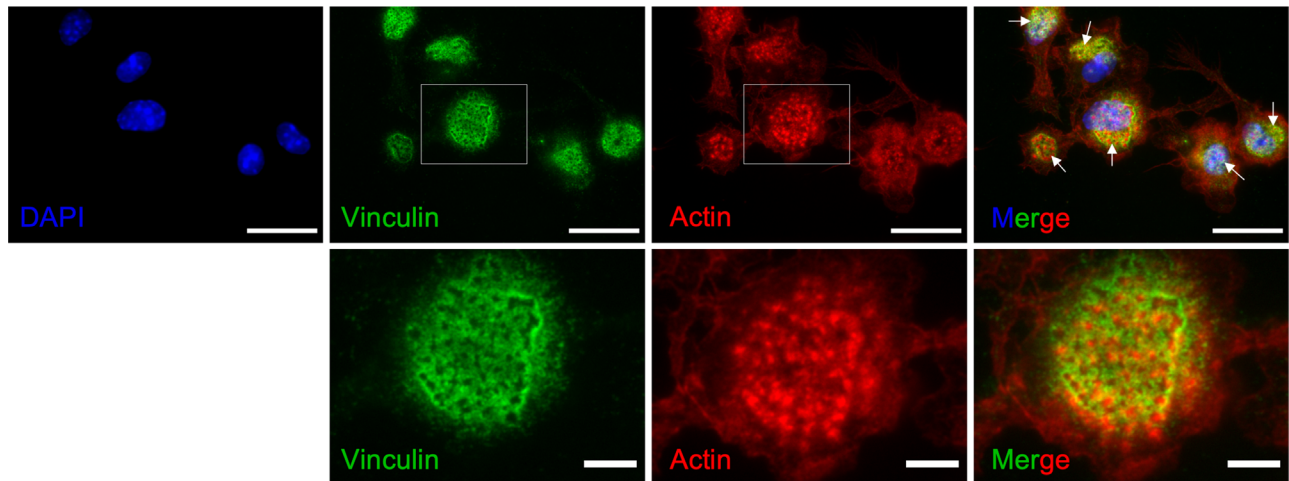
Podosomes act as adhesive and invasive structures that are particularly abundant in cells of the monocytic lineage including macrophages, dendritic cells, and osteoclasts but are also found in other cell types [56]. Podosomes were indeed first described in v-src-transformed fibroblasts as clusters of contact sites of cell-to-extracellular matrix (ECM) adhesion [57, 58]. Chen et al. later found that degradation of the ECM occurs at these contact sites and coined the term invadopodia [59]. Nowadays, it is widely accepted to refer to these structures in transformed tumor cells as invadopodia while referring to them as podosomes in normal cells. There are only subtle differences between invadopodia and podosomes, for example, regarding the durability of the structure,

whereas they can be clearly distinguished from other actin-based adhesive structures involved in cellular migration such as lamellipodia, filipodia, and focal adhesions (see [56] for an excellent overview). We follow this classification and therefore refer to this structure in DC throughout this guideline as podosomes.

Podosomes present as dot-shaped, micrometer-sized F-actin cores surrounded by an adhesive ring of adhesion plaque proteins, such as vinculin, paxillin, and talin, and anchored to the ECM by integrins [60, 61]. Meanwhile, it is well-established that podosomes are composed of more than 300 different components, highlighting their molecular complexity [61]. The characteristic bipartite appearance of the actin core and the adhesive ring facilitates their recognition in immunofluorescence microscopy (Fig. 9). However, in combination with fluorescent gelatin to assess its degradation, the detection of podosomes is limited to the identification of the actin cores.

A hallmark function of podosomes is the degradation of ECM that sets them apart from other actin-rich structures [56]. To this end, podosomes recruit matrix-lytic proteases, including matrix metalloproteases (MMP) and ADAMs (a disintegrin and metalloprotease). However, the role of proteolytic enzymes in other purposes of podosomes remains unclear. Beyond the above-mentioned features of adhesion and ECM degradation, podosomes are nowadays considered to fulfill additional functions [60]. The capability of cells to sense the rigidity and topography of the substrate through the podosomes has been demonstrated. Moreover, podosomes frequently form at protruding sites of the cell. It is thus hypothesized that podosomes are involved in the directional migration of cells by stabilizing otherwise transient protrusions. This may enable cells to transmigrate across layers of other cells, as shown for osteoclasts. Studies in DC have very specifically identified podosome-like protrusions as sites of antigen uptake [60].

The protocol presented here allows quantifying both podosome formation and matrix degradation simultaneously using fluorescent microscopy and thus can be used to study the molecular mechanisms of podosome biology and their function



**Figure 9.** Podosome staining and detection in DC. Bone marrow-derived DC were seeded on gelatin-coated coverslips and cultivated overnight before being fixed and stained for vinculin and actin to detect podosomes (arrows). Images at the top panel were taken with 100x magnification. The lower panel shows enhanced views of selected areas (white boxes) from the top images to highlight structural details of a podosome. Note that DAPI staining in the lower panel was excluded for better visualization of the podosome. Scale bar upper panel: 20  $\mu\text{m}$ ; scale bar lower panel: 5  $\mu\text{m}$ .

in DC. It is based on a protocol that used fluorescently-labeled gelatin as a matrix component for studying invadopodia formation by invasive tumor cells [62]. The workflow was modified and improved to meet the needs for the analysis of podosome formation and function in mouse bone marrow-derived DC in terms of gelatin coating, cell seeding, and data analysis. It may also serve as a kernel to extend it for studying further functionalities or of other DC subsets, including human monocyte-derived DC [63, 64].

## 5.2 Materials

### 5.2.1 Reagents

All chemicals and reagents are listed in Table 16.

### 5.2.2 Equipment

All equipment is listed in Table 17.

## 5.3 Step-by-step sample preparation

### 5.3.1 Coating of coverslips with fluorescently labeled gelatin

1. Prepare fluorescent gelatin stock solution of 1 mg/ml by adding 5 ml of sterile double distilled water (ddH<sub>2</sub>O) to the commercial vial following the manufacturer's instructions. For coating, a final working concentration of 0.2 mg/ml is used. Aliquots of the stock solutions can be stored at  $-20^{\circ}\text{C}$ .

2. Clean and prepare the surface of round 12 mm glass coverslips by soaking them in a mixture of EtOH/HCL (6:4; v/v) for 2 h with occasional shaking. Let them air dry. Dried coverslips can be stored for later use.
3. Sterilize the coverslips by immersion in 70% EtOH solution and allow to air dry.
4. Transfer the coverslips into a 24-well plate and add 500  $\mu\text{l}$  of poly-L-lysine (50  $\mu\text{g}/\text{ml}$  in ddH<sub>2</sub>O) per well. Incubate at RT for 20 min.
5. Wash coverslips three times with PBS (optionally, a water jet pump can be used for aspirating).

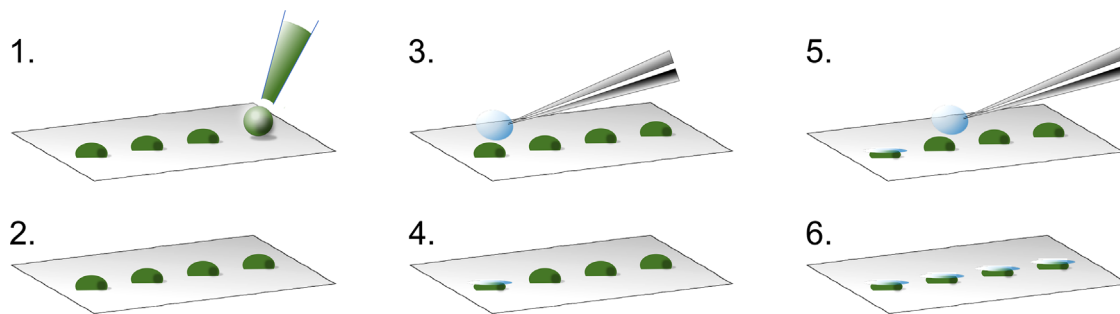
**Table 16.** List of chemicals and reagents

Reagent	Manufacturer	Catalog number
Ethanol (EtOH)	Merck	1009862500
Hydrochloride acid (HCL)	Merck	1090631000
Poly-L-Lysine, 0.1% in ddH <sub>2</sub> O	Sigma-Aldrich	P8920
Glutaraldehyde 25%	Polysciences Inc.	01909
Glycine	Merck	1042011000
Phosphate Buffered Saline (PBS)		
Fetal Calf Serum (FCS)	Gibco	
RPMI-1640	Gibco	21870076
GM-CSF	Peptotech	315-03
Gelatin from pig skin, Oregon Green 488 conjugate	Invitrogen	G13186
DAKO fluorescence mounting medium	Agilent	S3023
TNF $\alpha$ (optional)	Peptotech	315-01A

**Table 17.** List of equipment

Equipment	Manufacturer	Catalog number
24-well plate	Greiner Bio-One	662160
Round 12 mm diameter glass coverslips (Menzel™)	Fischer Scientific	11708701
Microscope glass slides (75×25 mm, frosted end)	Langenbrinck	03-0004
Parafilm	Bemis	PM-996
Forceps Dumont #5	Fine Science Tools (F.S.T.)	11252-20
Thermomixer set at 60°C		
Vacuum source (e.g. water jet pump) for aspirating (optional)		

6. Add 500  $\mu$ l of pre-chilled glutaraldehyde (0.5% in PBS) to each well and incubate on ice for 15 min.
7. During this incubation period, the Oregon Green 488-conjugated gelatin stock solution is heated to 60°C, diluted to the final working dilution of 0.2 mg/ml, and kept at 60°C.
8. Dispose of the glutaraldehyde properly and wash coverslips three times with PBS.
9. Place a sheet of parafilm on the bench and spot 50  $\mu$ l of fluorescent gelatin working solution on top. Leave enough space ( $\geq 1$  cm) between the drops. The drops will not disperse and flow into each other (Fig. 10).
10. Coat coverslips by placing them upside down on the fluorescent gelatin solution and leave them protected from light for 30 min at RT (Fig. 10).
11. Transfer the coverslips (with the top side up) back to a 24-well plate after the incubation period and let it dry for 10 min in the dark at RT.
12. Add 500  $\mu$ l of glycine (0.3 M in PBS; pH7,2) and incubate for 20 min at RT in the dark and wash coverslips three times with PBS afterward.
13. Transfer the plate with coverslips under the cell culture flow hood and transfer the coverslips into a sterile 24-well plate.



**Figure 10.** Steps for coating glass coverslips with fluorescent gelatin. 1.-2.) Spot 50  $\mu$ l of pre-warmed fluorescent gelatin working solution on top of a sheet of parafilm. 3.-6.) Place poly-L-lysine pre-coated coverslips upside down on the fluorescent gelatin solution. Leave them on for 30 min in the dark at RT.

Wash coverslips three times with sterile PBS. From this point on, the coated coverslips can also be stored in the dark at 4°C in the fridge for up to a week before seeding with cells. Continue to work under sterile conditions.

14. Add 1 ml complete RPMI medium (containing 10% FCS) to each well and equilibrate coverslips at 37°C for 30 min.

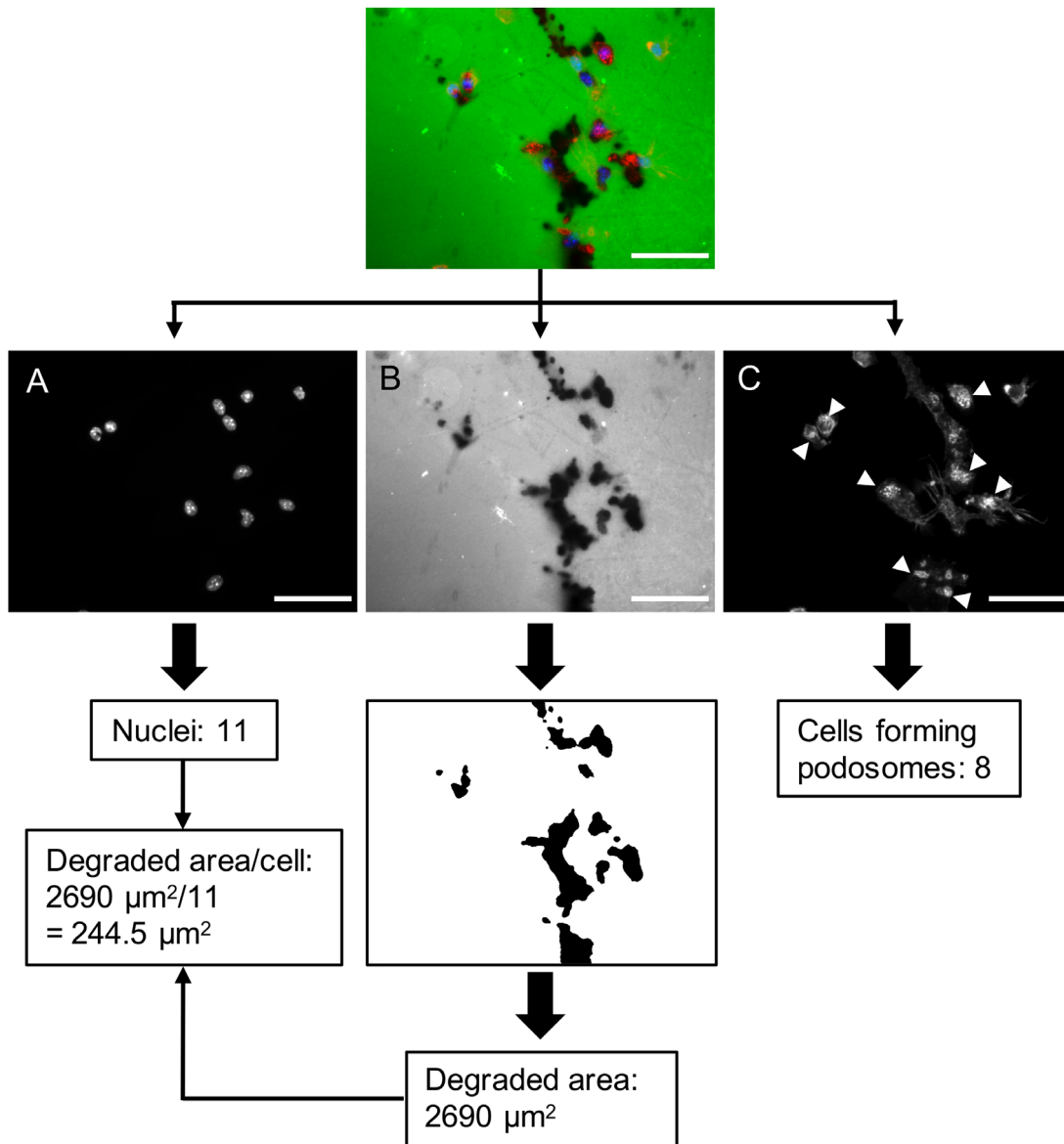
### 5.3.2 Preparation and seeding of DC and performing the gelatin degradation assay

1. Harvest bone marrow-derived immature DC from your cell culture and perform a complete RPMI medium change.
2. Count cells using a hemocytometer (or any other cell-counting device).
3. Resuspend cells at  $2 \times 10^5$  cells/ml in complete RPMI medium supplemented with GM-CSF (200 U/ml). Optionally add additional factors that you wish to investigate for their influence on gelatin degradation and the podosome formation by DC, such as TNF $\alpha$  (100 ng/ml), kinase inhibitors, or toll-like receptor ligands.
4. Aspirate the RPMI medium from pre-incubated coverslips and transfer 500  $\mu$ l of the DC solution ( $1 \times 10^5$  cells) per well.
5. Incubate for 8–16 h at 37°C.

### 5.3.3 Processing cells for podosome detection and fluorescence microscopy

All following steps can be again performed under non-sterile conditions.

1. At the endpoint, wash coverslips once with PBS and quickly fix cells by adding 500  $\mu$ l of paraformaldehyde (4% in PBS) per well and incubate for 15 min at RT in the dark.
2. Remove the paraformaldehyde and dispose properly and wash coverslips three times with PBS.
3. Add 500  $\mu$ l of blocking solution (3% BSA in PBS containing 0.1% TritonX-100) and incubate for 30 min at RT in the dark.
4. Remove the blocking solution and stain F-actin by adding 180  $\mu$ l Alexa Fluor 594-coupled Phalloidin diluted 1:2000 in 0.3%



**Figure 11.** Image analysis using Fiji and quantification of the degraded area per cell. Each channel from the same FOV is used to extract specific information. The blue channel (A) is used to quantify the number of cells per field by counting the nuclei. The green channel (B) is used to quantify the area of the degraded gelatin batches. The values obtained from the blue and green channels are used to calculate the degraded area per cell. The red channel (C) is used to quantify the number of cells that are forming podosome clusters (arrowheads). Scale bar: 50 μm.

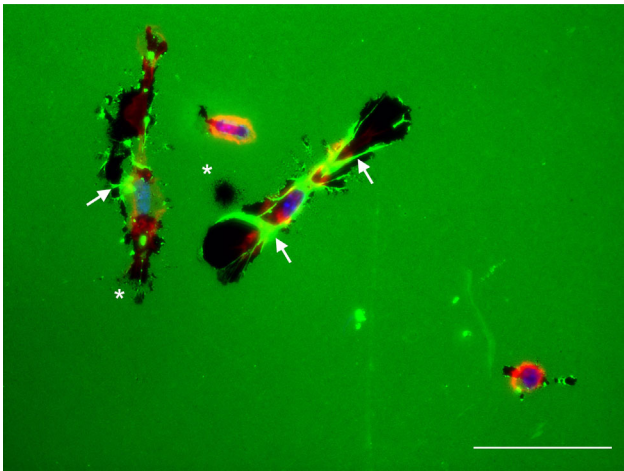
BSA/0.1% Triton X-100 in PBS solution per well and incubate for 1 h at RT in the dark.

5. Aspirate the staining solution and wash the coverslips three times with PBS.
6. Add 500 μl of DAPI (1:5000 in PBS) and incubate for 5 min at RT in the dark.
7. Wash coverslips three times with PBS.
8. Place a droplet of mounting medium on a glass slide and mount a coverslip by placing it upside down onto the droplet. Let it dry.
9. Slides can be stored in the dark at 4°C until imaging and for up to several weeks.

#### 5.3.4 Imaging of podosome formation and activity

1. Images of the slides are taken with a fluorescence microscope, typically using a 40x objective and equipped with filter sets for detection of the green (Oregon Green 488), the red (Alexa Fluor 594), and the blue (DAPI) fluorescence. Gelatin degradation causes loss of the green fluorescence. Accordingly, black areas in the otherwise homogeneous green background are indicative of gelatin degradation. The degraded areas do not have sharp edges and angles, but the boundaries appear rather blurred and sometimes speckled (Fig. 11 and 12).





**Figure 12.** Squeezing and pulling of fluorescently-labeled gelatin by DC on a non-precoated coverslip. Images taken in the blue (DAPI), green (Oregon Green 488-labeled gelatin), and red (Alexa Fluor 594-labeled phalloidin) channels are overlaid as in Fig. 11. Arrows point to squeezed gelatin deposits. Asterisks indicate degraded areas characterized by speckled boundaries. However, whether black areas are derived from gelatin degradation or squeezing is not clearly distinguishable. Scale bar: 50  $\mu\text{m}$ .

2. Podosomes are visualized in the red channel and identified as ensembles of F-actin dots on the ventral side of the cells in contact with the gelatin.
3. Quantification of podosome formation and activity is performed with the same set of images. To achieve representative and unbiased image data we recommend the following procedure. Divide the coverslips into imaginary top, middle, and bottom sections and take at least 5 images per section in all three fluorescence channels. Survey the sections using the blue (DAPI) channel and select areas where typically 4–6 individual nuclei can be identified in the field of view (FOV). Next, acquire images from the same area in the red and green channels. Then, move to another area using again the DAPI channel. This allows the most unbiased examination, as FOVs are then not selected primarily for strongly degraded areas or podosome-rich cells. Finally, each sample is represented by a set of at least 15 FOV, which in this case represent between at least 60 and up to 90 cells.

We do not recommend analyzing areas with more than 8 cells per FOV, since it is very likely that cells at such a high number clustered together, and thus degraded areas cannot be attributed to distinct cells. If more cells in such a cluster are capable of degrading gelatin this would result in a lower “degraded area fraction/cell” in the calculation (see 5.4 Data analysis below) due to the overlap of the degraded areas.

**IMPORTANT NOTE:** A characteristic of podosomes in primary cells is their fast turnover rate of only a few minutes (2–12 min) for assembly and disassembly of the structure. This is in strong contrast to invadopodia of tumor cells, which can persist for hours [60]. Consequently, at the time of fixing the cells on the coverslips, podosomes are not detectable on

all DC that might be capable of forming them. Accordingly, not all DC found associated with degraded areas will present podosomes.

## 5.4 Data analysis

All image analyses were performed using the image processing package Fiji ([www.imagej.net](http://www.imagej.net)).

1. To quantify podosome formation in DC, open an image with actin staining and the corresponding image with DAPI staining. Count the number of cells forming podosomes on each image of the actin staining and normalize it to the total number of cells by determining the number of nuclei in the DAPI staining. Note that some cells show more than one podosome cluster, while others show none (Fig. 9 and 11). Collected data from all images can be plotted as “percentage of podosome forming cells”.
2. The gelatin degradation is quantified by analyzing the proportion of the entire areas that correspond to degradation on a given image using the “area fraction” measurement option in Fiji. Like before, the “area fraction” value will then be normalized to the number of cells in each image as measured from the DAPI channel in the respective field (Fig. 11). The degraded area fraction can then be displayed as “percentage of normalized degraded area per cell”. However, we recommend converting the pixel size of the images into metric size (i.e.  $\mu\text{m}$ ), which allows plotting the data as, for example, degraded area/cell ( $\mu\text{m}^2$ ). The conversion from pixels to the metric unit depends on the optical settings of the camera and must therefore be determined individually for each device. For the conversion in Fiji go to: *Analyze* > *Set Scale*. On the new window enter the respective “Distance in pixels” and the corresponding “Unit of length” and confirm with “OK”.

For obtaining the “area fraction” value, select *Analyze* > *Set Measurement* > and select “Area fraction” from the new window. Open greyscale images of the green fluorescence showing the degraded areas as black spots within the gray background of the non-degraded gelatin (Fig. 11). Select *Image* > *Adjust* > *Threshold*. With opening the “Threshold” window the image will turn into a black and white image without any grayscale (Fig. 11). Degraded areas may appear as black spots over a white background or as white spots over a black background. Make sure that the option “Dark background” is not selected and press “Apply”. If the software displays black areas that are outside the actual degradation, for example, due to inhomogeneities of the gelatin coating, the threshold has to be adjusted manually using the horizontal scroll bars in the “Threshold” window. When the dark areas are representative of the real degradation observed in the original image press “Set”. Record the “area fraction” by selecting *Analyze* > *Measure*. The data are reported in a “Result” window and can be saved and transferred to a spreadsheet program.

3. Treatment conditions of cells could potentially influence the adhesion of DC to the gelatin coating. Reduced adhesion may thus result in reduced podosome formation and lower degradation activity. The spread of the cell body on the gelatin coating can be used as a readout of the net adhesive properties of the cells. The area that is covered by the cell spreading can be assessed using the same procedure as for determining the “area fraction” of degraded gelatin (see step 2) but by using the red channel images of the actin staining. Again, presenting the data in square micrometers is recommended.
4. Apply the appropriate statistical analysis to determine significant differences among various conditions.

### 5.5 Pitfalls

One should be careful not to invert the top side after coating.

We also recommend the use of very fine forceps, such as Dumont #5 (or equivalent) to avoid breaking the glass coverslips when removing them from the 24-well plates.

In addition to degrading the gelatin some cells may also pull the gelatin together (Fig. 12). Since the effect of such squeezing and pulling cannot be distinguished from the effect of degradation, in such a case the quantification of gelatin degradation would be falsified. It was assumed that too strong adhesion of the cells is the reason for this, and accordingly, means for reducing the adhesion of cells to gelatin have been suggested, including reducing serum concentration in the medium or increasing gelatin concentration to 1 mg/ml [62]. **IMPORTANT:** After we had established precoating of coverslips with poly-L-lysine we also noticed that the squeezing and pulling effect was minimized and thus we highly recommend this step in our protocol (see also 5.6).

The ability to form podosomes and their kinetics of assembly and disassembly vary depending on the state of differentiation and maturation of DC [60, 63, 64]. Maturation by TNF $\alpha$  or toll-like receptor signaling, i.e. using LPS will lead to different results. Maturation with LPS showed a different kinetic of podosome formation in DC dependent on the tissue source, i.e. BM-derived versus spleen-derived DC [63]. Moreover, LPS stimulation results in a complete loss of podosome formation within hours [64]. It is therefore essential to ensure that endotoxin-free reagents are used.

### 5.6 Top tricks

We recommend using 12 mm round glass coverslips together with a 24-well plate in combination with a water jet pump for all the washing steps. This allows convenient coating and use of up to 24 gelatin-coated coverslips in one experiment.

Precoating with poly-L-lysine proved to be advantageous in several respects. We found that a more homogeneous coating could be achieved with the fluorescently labeled gelatin. This could also be one reason for preventing the squeezing and pulling of the gelatin coating.

## 6 The under-agarose migration assay: a simple method to visualize and quantify mouse DC migration in confined 3D-like microenvironments

### 6.1 Introduction

On their migration routes dendritic cells (DC) encounter complex 3D-microenvironments like the interstitium [65]. The cellular principles, molecular mechanisms, and mechanobiology [66–68] of DC migration in 3D-microenvironments can be discovered *in vitro*, such as in collagen matrices [69], microchannels [70, 71], and forests of micro-pillars [72].

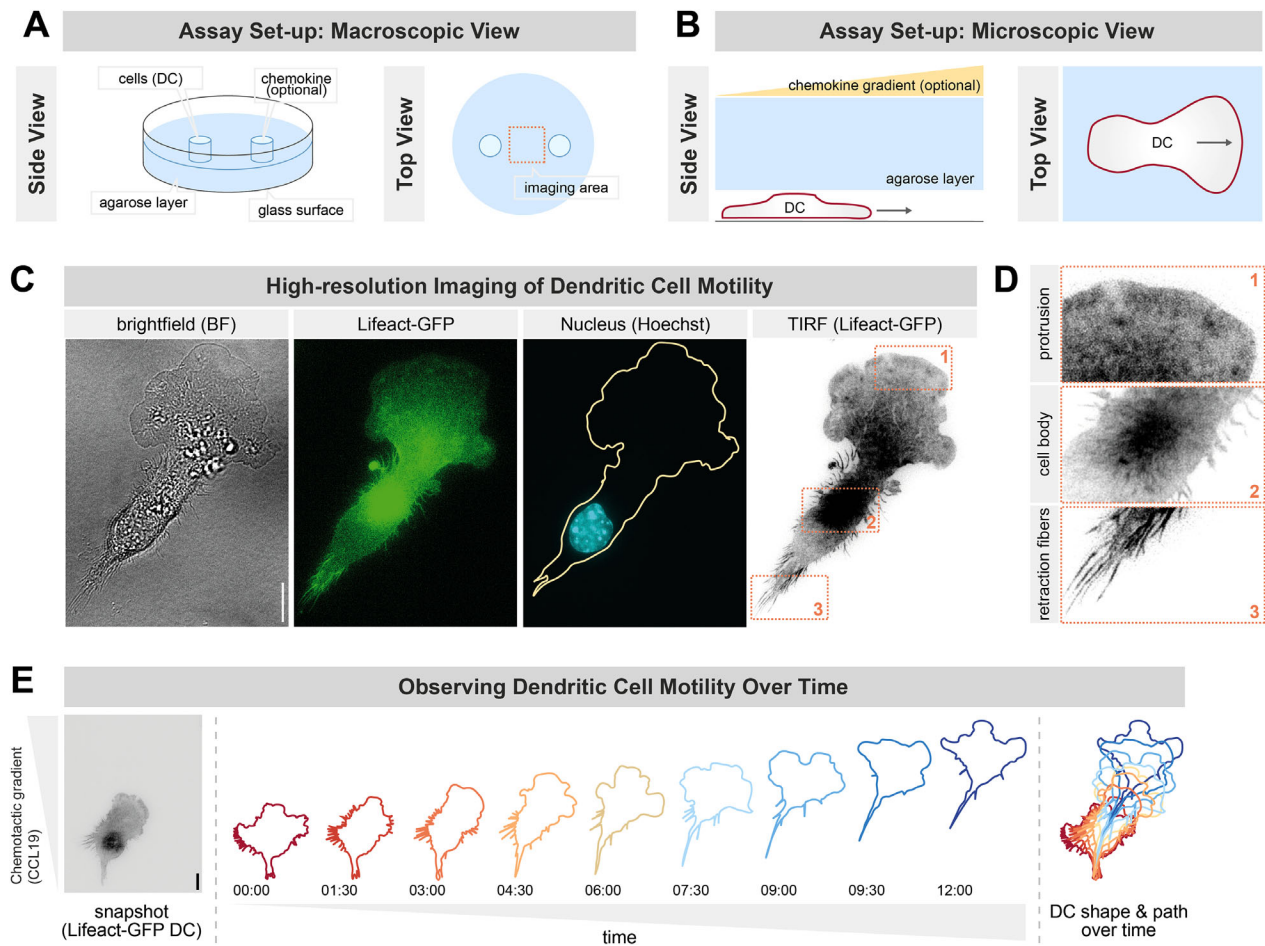
In this section, we describe the ‘under-agarose’ assay [73] as an additional, highly simple and versatile [74] method to visualize and quantify DC migration in confined 3D-like microenvironments. The assay (i) can be easily established without micro-engineering equipment, (ii) can be combined with live-cell, immunofluorescence, and advanced imaging, (iii) is compatible with patterned substrates, and (iv) is suited to investigate subclasses of immune cells like DC subtypes.

In the ‘under-agarose’ assay, DC migrate between a layer of agarose and the substrate (Fig. 13A, B). Thus, DC are highly confined and interact with their local microenvironment in three dimensions (in XYZ). Consequently, the locomotion mode is closely related to migration in 3D [75]: whereas DC on two-dimensional (2D) substrates employ a fundamentally different migration mode that is adhesive and integrin-dependent [76], DC in the ‘under-agarose’ assay can migrate integrin-independently without adhesions [77, 78], comparable to low-adhesive *in vivo* migration in the interstitium [76, 79, 80].

While mimicking cellular confinement in 3D, DC in the ‘under-agarose’ assay migrate directly on the surface close to the objective. This characteristic enables high-resolution imaging of sub-cellular processes like cytoskeletal dynamics, for instance by TIRF (total internal reflection fluorescence) [77, 78, 81] microscopy (Fig. 13C-E), spinning-disc microscopy [82], and FRET (fluorescence resonance energy transfer) [83]. Moreover, the assay is compatible with immunofluorescent staining [82, 84] and thus STED (stimulated emission depletion) microscopy, as well as CLEM (correlated light and scanning electron microscopy) [85] and light-based uncaging of caged chemoattractants [86].

DC in the ‘under-agarose assay’ migrate not only closely but also perpendicularly to the imaging plane. Thus, multiple cells can be imaged in low magnification to quantitatively analyze migration parameters like velocity and persistence [87]. Performing the experiment in multi-well dishes with glass-bottoms allows the comparison of different conditions in the same experiment (e.g., wild-type versus knockout).

Notably, the assay does not require any specialized micro-engineering equipment (all reagents and equipment are listed in Tables 18 and table 19, respectively) and thus can be established in every laboratory (Fig. 14A). We here describe the basic protocol of the ‘under-agarose’ assay. However, the assay is highly versatile and thus can be adapted to the respective research question



**Figure 13.** The under-agarose assay: imaging mouse dendritic cell (DC) motility in confined 3D-like microenvironments. (A, B) Scheme of the setup of the under-agarose assay. DC (or other cell types) are injected (see also Fig. 14) underneath a layer of agarose (light blue). Thereby, DC migrate highly confined and with a flat cell shape between the agarose layer (on top) and the glass surface (below). By stamping a hole as a chemokine reservoir (see also Fig. 14), the assay can optionally be employed to study DC chemotaxis. (C) Representative DC (Hoxb8-derived mouse DC encoding the actin marker Lifact-GFP) migrating under-agarose and imaged by brightfield (BF), epifluorescence (Lifact-GFP in green; the nuclear stain Hoechst in cyan), and TIRF microscopy (Lifact-GFP in gray). The scale bar equals 10  $\mu\text{m}$ . (D) Zoom into the exemplary regions 1–3 highlighted (orange) in c. (E) Representative Hoxb8-derived mouse DC (left panel) imaged over the time course of 12 min (middle panel), showing DC shape and locomotion (right panel). The scale bar equals 10  $\mu\text{m}$ .

(Fig. 14B). Examples include (i) integration of chemokine sources to study chemotaxis or chemokine competition [88], (ii) modulation of the mechanical load by the microenvironment [85], (iii) combination with ligand-patterned substrates [80, 89], and (iv) combination with substrate topographies like nano-ridges [85, 90, 91]. Moreover, the assay is compatible with other leukocytes like neutrophils [92], neutrophil-like HL-60 [93] and PLB-985 cells [94], T cells [90, 95, 96], and non-leukocytes like endothelial cells [97], cancer cells [98, 99], zebrafish progenitor cells [100], and the single-cell amoebae *Dictyostelium* [101]. As only small cell numbers are required, the assay can be combined with leukocyte-pathogen studies [102] and immune cell isolations from patients with human immunodeficiency [103].

In summary, the ‘under-agarose’ assay is a simple but highly versatile assay to investigate the cellular principles and molecular mechanisms of DC migration in confined 3D-like microenvironments.

## 6.2 Materials

### 6.2.1 Reagents

Necessary reagents are listed in Table 18

### 6.2.2 Equipment

Necessary equipment is listed in Table 19

## 6.3 Step-by-step sample preparation

### 6.3.1 Preparation of the ‘under-agarose’ assay

1. Preparation of reagents: Prepare 2 $\times$  HBSS by diluting 100 ml of 10 $\times$  HBSS in 400 ml of ddH<sub>2</sub>O. As a precaution, the

**Table 18.** Reagents and solutions

Reagent	Manufacturer	Catalogue Number
RPMI 1640, no phenol red	Gibco	11835063
Fetal Calf Serum (FCS)	Gibco	
10,000 U/ ml Penicillin- 10 mg/ ml Streptomycin (P/S)	Sigma-Aldrich	P0781
2-Mercaptoethanol (50 mM)	Gibco	31350-010
UltraPure™ Agarose	Invitrogen	16500-500
10× Hank's Balanced Salt Solution (HBSS)	Thermo Fisher Scientific	14065056
CCL19 (optional)	bio-technie	440-M3
NucBlue (optional)	Invitrogen	R37605

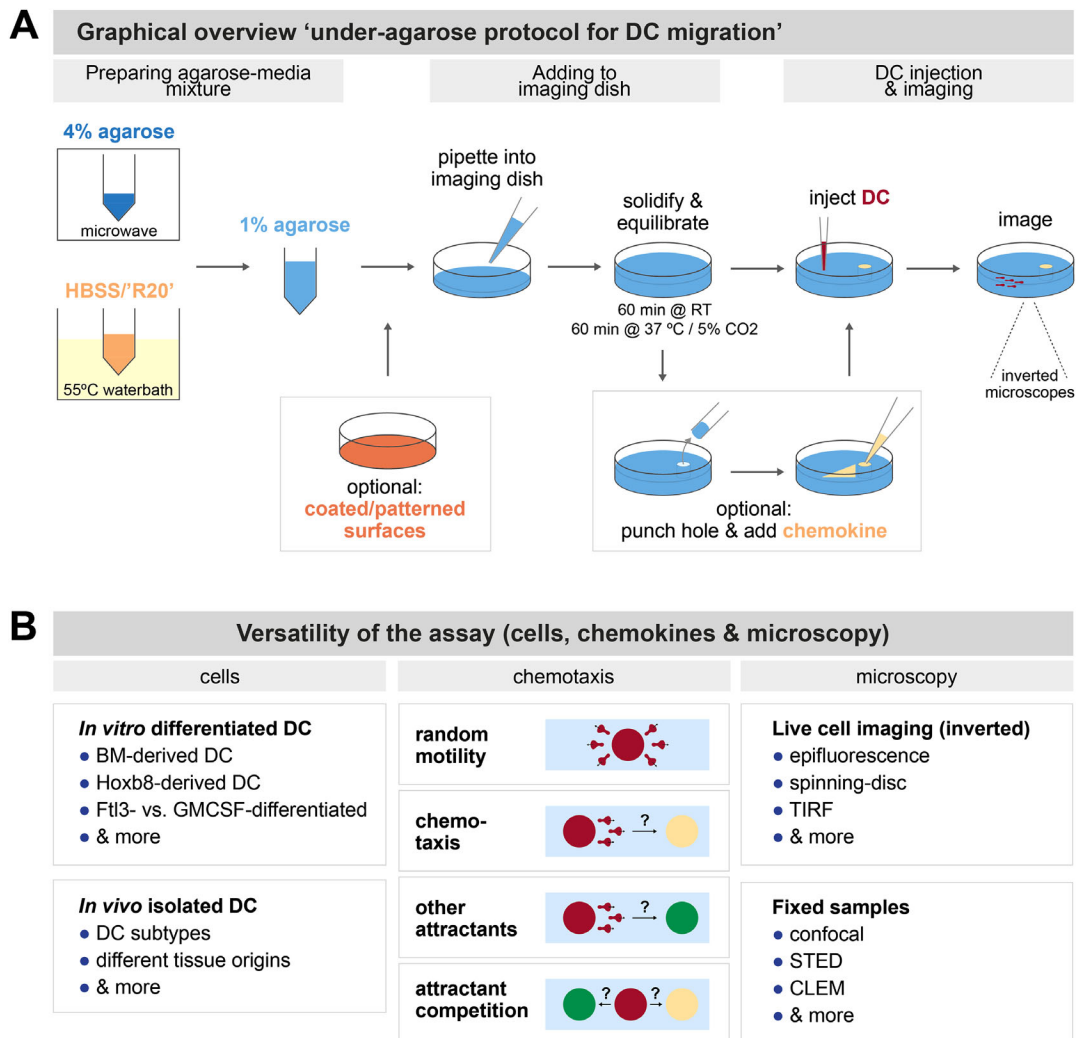
solution may be filter-sterilized and stored at 4°C. Prepare RPMI-1640 + 20% FCS (R20) by adding 120 ml FCS, 6 ml

**Table 19.** Necessary equipment

Equipment	Manufacturer	Catalogue Number
µ-Slide 8 Well Glass Bottom	Ibidi	80827
35 mm glass bottom dish	Greiner Bio-One	627860
Harris Uni-Core puncher 2.0 mm (optional)	Sigma-Aldrich	WHAWB100076
Water bath, adjustable to 55°C		
Microwave		

P/S and 660 µl 2-Mercaptoethanol to 500 ml RPMI-1640. R20 and 2× HBSS can be stored at 4°C for later use.

2. Decide for the final agarose concentration (influencing the stiffness of the agarose gel [85]) and calculate the



**Figure 14.** Graphical overview & versatility of the protocol to visualize and analyze DC migration. (A) Graphical step-by-step protocol to investigate DC migration in the under-agarose assay (see the main text for experimental details). (B) Graphical overview on the versatility of the under-agarose migration assay.



ingredients accordingly. We routinely use a final agarose concentration of 1% when investigating DC: calculate the ingredients in a 1:2:1 ratio (2× HBSS:R20:water-agarose) for a final agarose solution with a concentration of 1% agarose (e.g. use 5 ml 2× HBSS, 10 ml R20, and 5 ml 4% agarose for a total volume of 20 ml). Note that the components of the agarose solution have to be added in a sequential order (steps 3–5).

3. Start to prepare the agarose solution by first mixing 2× HBSS and R20 in a 50 ml falcon tube and pre-equilibrate in a water bath to 55°C (30 min of pre-equilibration; see also Fig. 14).
4. Dissolve the agarose powder in ddH<sub>2</sub>O in another 50 ml falcon tube (e.g., at an initial concentration of 4% agarose to achieve a final concentration of 1% agarose). Heat up by using a microwave until the agarose is fully dissolved: heat shortly, shake/vortex the tube and heat up again in the microwave; repeat these steps at least three times until the agarose is completely dissolved (important: (i) mildly unscrew the falcon lid and (ii) prevent overheating by reducing microwave power).
5. Immediately pour the 55°C pre-equilibrated 2× HBSS/R20 mixture (step 3) to the dissolved agarose (step 4); see also Fig. 14.
6. Immediately pipette the mixture from step 5 into an imaging-suitable dish with glass bottom (e.g., use 2.5 ml for a 3 cm glass bottom dish or 300 µl/well for an 8-well IBIDI µ-Slide).
7. (Optional): Usage of coated glass-coverslips: If you use a coated glass surface (e.g. PEG-, Fibronectin-, poly-L-lysine- or ConA-coated), then let the 2× HBSS/R20/water-agarose mixture (step 5) cool down to 37°C before adding it onto the coated coverslips to prevent inactivation of the coating.
8. (Optional) Inhibitor experiments: the under-agarose assay can be combined with pharmacological inhibitors. To prevent inactivation of the inhibitors, let the 2× HBSS/R20/water-agarose mixture (step 5) cool down to 37°C until you add the inhibitor in the respective concentration into the HBSS/R20/water-agarose mixture. Do not forget to add the inhibitor also in the later steps to the cells and to the chemokine to ensure a homogeneous concentration of the inhibitor.
9. Solidify the agarose mixture for 1 hour (h) at room temperature to generate a solid agarose gel.
10. Equilibrate the entire set-up at 37°C and 5% CO<sub>2</sub> in a humidified cell culture incubator for 1 h.
11. (Optional pause point): We recommend to continue with the next step (injection of DC) after equilibration of the set-up for 1 h. Yet, the set-up can also be incubated for longer time in the incubator (e.g., 2 to 5 h). Thus, this step represents an optional pause point, if necessary. However, we do not recommend long incubation (e.g., overnight), as changes in humidity can influence the assay during long incubation periods (e.g., we occasionally observed (i) drying-out of the agarose layer, if the humidity is too low in the cell culture incubator, and (ii) floating of the agarose layer, if the humidity is too high in the cell culture incubator).

### 6.3.2 Injection of DC into the ‘under-agarose’ assay

1. (Optional) Combine the under-agarose assay with chemotaxis: Stamp a hole into the agarose layer (e.g. by using a biopsy puncher with 2 mm diameter) and fill the hole with the respective chemokine (e.g. 15 µl of 2.5 ng/µl CCL19 diluted in medium; see also Fig. 14).
2. Harvest DC (e.g. bone-marrow derived LPS-matured DC) from your culture and count the cells.
3. Inject 20,000 DC dissolved in 1 µl media between the glass-coverslip and the agarose-layer by using smallest pipette tips. Be careful to push the pipette tip entirely through the agarose layer until you reach the glass surface: hold the pipette straight and only then release the 1 µl of cell solution while still pushing mildly against the glass slide. If you are combing the assay with a chemokine gradient (step 1), inject the cells in 5 mm distance to the chemokine hole 1 h after adding the chemokine (this allows the chemokine gradient to be established by diffusion).
4. (Optional pause point): Before imaging, we recommend to incubate the DC upon injection for 2 h below the agarose layer. Depending on the research questions, longer incubation periods in the range of several hours are also possible. Thus, this step represents an optional pause point, if necessary. Yet, we do not recommend incubation for very long time periods (e.g., overnight or for several days), as long-term confinement might induce changes in gene expression and alterations in the DC differentiation/maturation status (see also ‘pitfalls’, point 4).

### 6.3.3 Live-cell Imaging of DC in the ‘under-agarose’ assay

1. Use an inverted microscope with an incubation chamber heated to 37°C and supplied with humidified 5% CO<sub>2</sub>. Setup the microscope and the microscope software according to your research question.
2. (Optional) DIC microscopy: as the agarose-layer interferes with DIC microscopy, adjust the amount of the 2× HBSS/R20/water-agarose mixture to only result in a thin layer of agarose.
3. (Optional) Instead of live-cell imaging, the under-agarose assay can be combined with fixation and thus immunofluorescence staining (see e.g., [82, 84]). Note that the fixation agent has to diffuse through the agarose layer; we recommend to add 37°C pre-warmed 4% PFA followed by incubation for 1 h at 37°C.

## 6.4 Data analysis

DC migrating in the ‘under-agarose’ assay can be analyzed by multiple open-source- and commercial-image analysis software packages. Below we describe the analysis of basic DC migration parameters using the image processing package Fiji/ImageJ [104, 105] ([www.imagej.net](http://www.imagej.net)).

1. To quantify basic cell migration parameters (e.g., migration speed or directionality), cells can be tracked and analyzed using the Chemotaxis and Migration Tool [106] plug in. If a live-cell compatible nuclear staining is included (e.g., Hoechst), cells can be tracked automatically using the TrackMate [107] plug in (select *Plugins* > *Tracking* > *TrackMate*). Detailed information on how to use TrackMate can be found here [TrackMate-manual.pdf](#). If the experiment was performed without fluorescent staining, cells can be tracked manually by selecting *Plugins* > *Tracking* > *Manual Tracking*. The tracking data are reported in a 'Result' window and include information on track number, slice number, as well as X and Y coordinates. Import the respective tracking data table in the Chemotaxis and Migration Tool [106] plug in (select *Plugins* > *Chemotaxis Tool* > *Import data*) and initialize the data under 'Settings' by specifying X/Y units and time intervals according to your imaging setup. If desired, tracks with a distinct track length can be specifically selected for downstream analysis by adjusting the parameters for 'Number of slices' accordingly. Add the dataset and apply the settings. Once initialized, tracks can be plotted, for example, on an X/Y diagram or as rose plot. For obtaining additional parameters such as directionality, velocity, accumulated or Euclidian distance, select the 'Statistic feature' tab.
2. To analyze the cell shape, the 'Particle Analyzer' can be employed: Open an 8-bit greyscale image of a fluorescent staining visualizing the entire cell. Select *Image* > *Adjust* > *Threshold*. Thereby, the image is converted into a binary image. Make sure that "Dark background" is selected. If necessary, adjust the threshold manually using the scroll bars until the binary image is representative of the original greyscale image and press "Set". Select *Analyze* > *Analyze Particles*. Adjust size parameters according to cell size and confirm with "OK". The data are reported in a "Summary" window and include parameters describing cell shape, e.g., area, major and minor axis, and circularity. If it is not possible to generate a representative binary image due to a high background signal, we recommend performing segmentation with Ilastik [108], a free open-source software for image classification and segmentation.

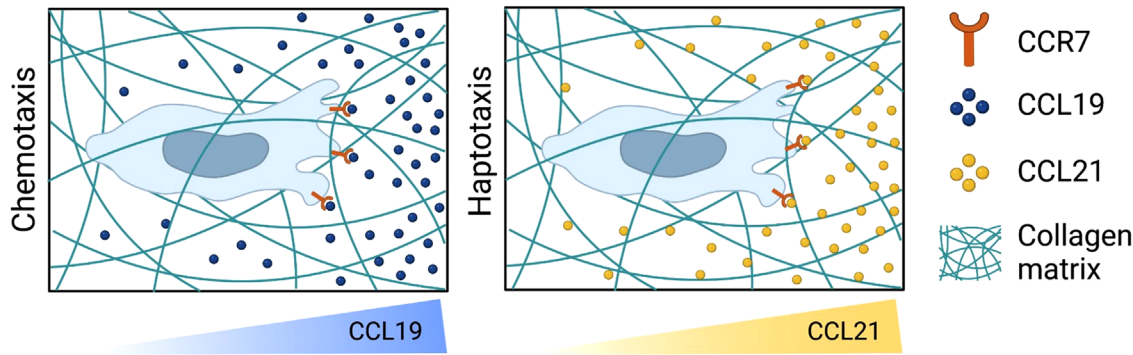
## 6.5 Pitfalls

1. Desiccation/humidification of the agarose gel will affect the degree of cellular compression. Thus, in each step of the protocol one should take care that the agarose neither dries out nor gets too watery (see 'top tricks' 1–4).
2. Inhomogeneous dissolving of the agarose powder would result in agarose clumping, thereby effecting light scattering and thus imaging properties (see 'top tricks' 5–7). Similarly, a very thick layer of the agarose gel would reduce the quality of the optical properties.
3. Be careful to not destroy coated surfaces or pharmacological inhibitors by temperatures above 37°C (see 6.3.1, points 7 and 8).

4. We typically employ the 'under-agarose' assay for short-term investigations (2 to 5 h) and with LPS-matured mouse DC. When employing non-matured DC or DC from tissues, it is critical to consider that changes in gene expression patterns and in the differentiation/maturation status can occur when DC are extracted from tissues. Thus, we recommend to either (i) only perform short-term investigations in the range of a few hours directly upon DC isolation, or (ii) carefully control the differentiation/maturation status when performing long-term investigations. Similarly, for long-term investigations we recommend to carefully exclude any endotoxin contaminations that could alter the DC maturation status.
5. DC are highly confined in the under-agarose assay. Thus, DC typically drag their nucleus – the largest and stiffest organelle – rather at the back of the cell, whereas DC in less confining microenvironments position their nucleus rather to the front of the cell body directly behind the lamellipodium.
6. DC migration is typically highly sensitive to temperature and CO<sub>2</sub>. Thus, we highly recommend using microscopes with incubation chambers pre-heated to 37°C for at least 1 h and supplied with humidified 5% CO<sub>2</sub>.

## 6.6 Top tricks

1. Be careful when dissolving the agarose powder in water by using a microwave: too frequent/long heating periods could result in water desiccation and thus a higher final agarose concentration than planned.
2. We recommend using sufficiently large volumes (at least 5ml) to dissolve the agarose powder in water. Otherwise, the water might desiccate too quickly and thus would again alter the final agarose concentration. Always use tubes/beakers 2–4 times the volume of the agarose solution to be prepared.
3. We recommend ensuring that agarose gels are equilibrated at 37°C always for the same duration (1 hour) for every replicate, since longer incubation may lead to desiccation of agarose gels and thus would alter the degree of cellular confinement by the agarose.
4. For live-cell imaging, we recommend adding a reservoir of water around the sample to avoid desiccation of the agarose gel (even if humidified CO<sub>2</sub> is used). For example, when using multi-well plates, we recommend adding water into the empty space between the wells.
5. Prepare fresh agarose gels prior to every experiment. On the same day, prepared agarose gels may be kept at room temperature for a few hours before start of experiment.
6. Allow agarose powder to hydrate in cool H<sub>2</sub>O for a few minutes before heating in microwave. This allows dispersion of agarose powder and easier dissolution.
7. Avoid too vigorous vortexing of agarose to prevent particles from sticking to the wall of the tube, as this may result in undissolved agarose particles in the final product.



**Figure 15.** Scheme showing the guidance mechanisms induced by CCR7 activation. CCL19 stimulates DC chemotaxis (left panel) by generating a soluble gradient. CCL21 binds to the extracellular matrix generating an adhesive gradient, stimulating DC haptotaxis along immobilized chemokines.

8. If you encounter air bubbles after combining 2× HBSS with dissolved agarose, then return the mixture to the 55°C water bath for 2 min to let bubbles rise to the upper surface.
9. Glass-bottom imaging dishes are commercially available in different sizes (e.g., 8-well  $\mu$ -Slides from IBIDI, 3.5 cm dishes from MatTek, or 3 cm glass bottom dishes from Greiner). Alternatively, custom-made dishes can be used, enabling the use of multi-well dishes like 6-wells: drill circular holes (e.g., made by the institute's workshop) into the bottom of the plastic dish and cover the hole with a cover glass suitable for microscopy (glued to the dish by e.g., paraffin).

## 7 Quantification of DC migration in 3D collagen matrices

### 7.1 Introduction

Dendritic cells (DC) patrol tissues in search for danger signals in health and disease [65, 109]. These signals include damage-associated molecular patterns (DAMPs), such as extracellular adenosine triphosphate (ATP) [110, 111]. In addition, DC are activated by pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS) [87, 110-113]. Interestingly, ATP and LPS activate different calcium signaling pathways, but both require actin cytoskeleton reorganization to increase DC migration [68, 114], revealing the functional convergence of DAMPs and PAMPs [110, 115]. Moreover, both danger signals trigger the expression of chemokine receptors (i.e. C-C chemokine receptor type 7, or CCR7), which allow DC trafficking toward the lymphatic vessels [116]. The overexpression of CCR7 after encountering DAMPs and PAMPs highlights the relevance of chemokine sensing and DC migration to initiate the adaptive immune response [109]. Consequently, the distortion of both DC migration and CCR7 signaling is associated to diseases [116, 117].

CCR7 binds two chemokines of the same family, but that have different properties [116]. These chemokines are C-C

motif chemokine 19 (CCL19) and C-C motif chemokine 21 (CCL21), which interact differentially with the extracellular matrix (Fig. 15), but in both cases induce directional migration [88, 118]. CCL19 remains preferentially in soluble form (Fig. 15, left), and therefore it induces chemotaxis [88, 118]. On the other hand, CCL21 released from the lymphatic vessels binds to the extracellular matrix [88, 118], and establishes an adhesive gradient (Fig. 15, right). The latter is named haptotaxis, which refers to the sensing of surface-bound chemical cues [88, 118].

In the recent years the number of techniques to study spontaneous DC migration has increased [68, 70, 71, 88, 111, 118-120]. Oppositely, the study of directional DC migration has limited possibilities and still one the most used techniques to study chemokine-induced migration is the transwell assay. However, despite its advantages this technique does not allow the monitoring of cell behavior dynamics, and it only evaluates the transmigration capacity of the cells. Then, the use of microscopy for live cell imaging emerges as a powerful tool to evaluate DC migration. In particular, in order to mimic the topology of the complex 3D microenvironments in which DC navigate in the tissue [88, 118], the use of 3D matrices has emerged as great alternative [121].

In this section we describe, a simple method to visualize DC migration in 3D microenvironments. In addition, we take advantage of the features of CCL19 and CCL21, to study both chemotaxis and haptotaxis. The current section shows the protocol to study mouse DC, but the same protocols can be used to study human DC.

### 7.2 Materials

#### 7.2.1 Reagents

Necessary reagents are listed in Table 20.

#### 7.2.2 Equipment

Materials for imaging of collagen assay are listed in Table 21.

**Table 20.** List of reagents specific for DC culture and collagen assay

Reagent	Manufacturer	Catalog Number
BMDC		
Iscove Modified Dulbecco Media (IMDM) Glutamax	ThermoFisher	31980048
2-Mercaptoethanol (50 mM)	ThermoFisher	31350010
Fetal Bovine Serum (FBS)	bioWest(VWR)	S1810-500
Penicillin/Streptomycin (P/S)	ThermoFisher	15070063
GM-CSF	R&D Systems	451-ML/CF
IL-4	R&D Systems	404-ML/CF
DPBS	ThermoFisher	14190169
EDTA	ThermoFisher	2187281
Lipopolysaccharides from Salmonella enterica serotype typhimurium	Sigma	L6S11
Collagen I, Rat Tail	Corning®	354249
Collagen I, bovine	Neutragen Advanced Biomaterials	5010
NaOH (Sodium hydroxide solution)	Sigma (Merck)	1091371003
NaHCO <sub>3</sub> (Sodium bicarbonate solution)	Sigma (Merck)	S8761-500ML
Silicon paste	Avantor	291-1220
rmCCL19/MIP-3β	R&D Systems	440-M3/CF
rmCCL21/6Ckine	R&D Systems	457-6C/CF
DPBS (10×)	ThermoFisher	14200075

**Table 21.** Materials specific for imaging of collagen assay

Equipment	Manufacturer	Catalogue Number
Sterile glass bottom dishes, 35.5×10mm	wpi-europe	FD35-100
Glass coverslips	Mercateo	CB00120RA120MNZO

## 7.3 Step-by-step sample preparation

### 7.3.1 Generation of bone marrow derived mouse dendritic cells

- DC precursors from mice bone marrow were differentiated into bone marrow-derived DC (BMDC) with cytokines (50 ng/ml GM-CSF and 50 ng/ml IL-4). The cultures were maintained in IMDM media supplemented with FBS (10%), Penicillin/Streptomycin (100 U/ml), and 2-mercaptoethanol (50 μM) for 9–10 days, as previously described [111]. For a detailed description of reagents see Table 20.
- To activate DC and induce their maturation and expression of CCR7, it is possible to use a short pulse (30 min) of danger signals and analyze the cellular behavior after 16 h (overnight). For ATP concentration >100 μM ATP trigger full maturation,

it is recommended to use 500 μM to observe an increase in the speed of migration as previously described [111]. In the case of LPS it is recommended to use 100 mg/ml, although lower concentrations (>10 ng/ml) also trigger DC maturation after overnight treatment.

### 7.3.2 Preparation of collagen gels

In this article, we present a protocol to prepare two different types of collagen matrices, which have different features as described in the sections below.

#### 7.3.2.1 Bovine collagen type I.

- The preparation of the mixture with bovine collagen is performed on ice, in order to prevent early polymerization. Thus, add 13.5 μl of the media to a 500 μl tube. For a detailed description of reagents see Table 21.
- Carefully add 102 μl of bovine collagen type I to the media without mixing (stock of 3 mg/ml).
- Add 60 μl of cells (cell suspension contains  $2.7 \times 10^6$  cells/ml) and homogenize carefully.
- Finally, add 6.5 μl NaHCO<sub>3</sub> (stock solution 7.5 %) and mix gently to avoid the formation of air bubbles in the solution.

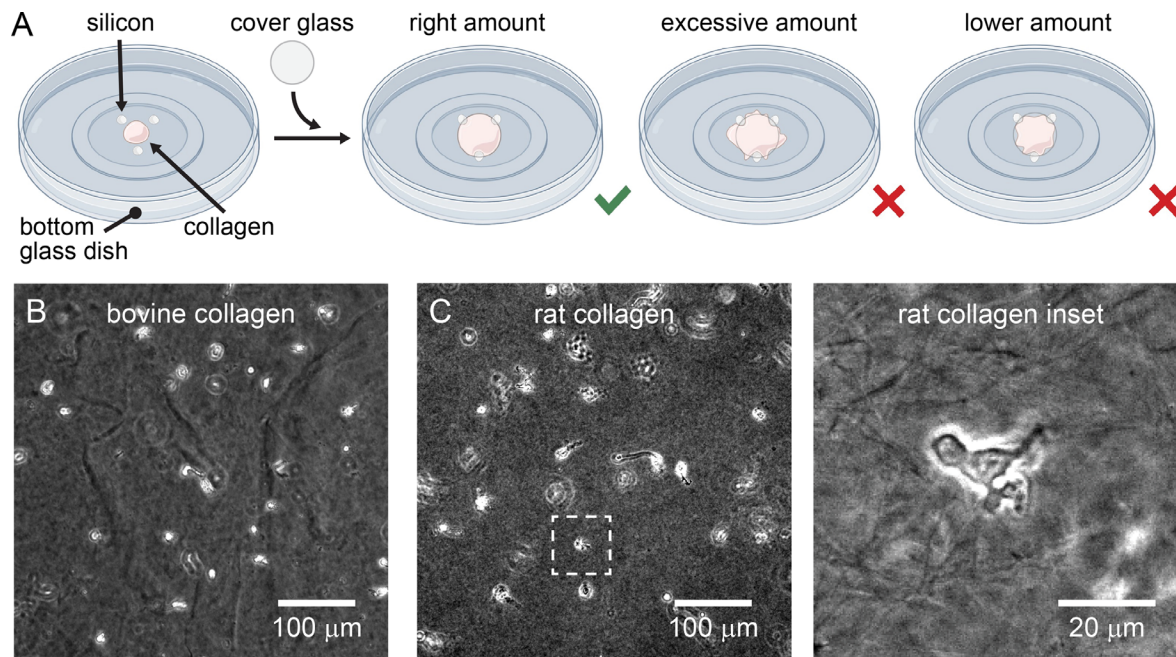
#### 7.3.2.2 Rat tail collagen type I.

- First, add 12.5 μl of PBS (10×) to a 500 μl tube.
- Place 29.4 μl media on top. Then carefully add 34.2 μl of rat collagen (stock of 3 mg/ml).
- Add 45 μl of cells (cell suspension contains  $2.7 \times 10^6$  cells/ml)
- Finally add 3.9 μl NaOH (1 M) to start the polymerization. Mix the sample carefully until it is homogeneous.

#### 7.3.2.3 Preparation of experiment setup.

- Prepare a bottom glass Petri dish (Fluorodish) by placing 3 drops of silicone forming an equilateral triangle (Fig. 16A). For a detailed description of the materials to prepare this and following steps, see Table 21.
- Add 40 μl of the collagen/cell mixture (from either 3.2.1 or 3.2.2) to the center of the three silicone drops.
- Using tweezers place a 12 mm circular glass coverslip on top of the collagen/cell mixture so that the border sticks to the 3 silicone droplets (Fig. 16A). Push the coverslip gently so the silicone is stuck in the coverslip.
- Prevent the collagen/cell mix from leaking outside the border of the coverslip (Fig. 16A). In addition, the collagen/cell mix should not leave empty spaces below the surface of the coverslip to prevent the drying of the sample (Fig. 16A).
- Incubate the collagen/cell mix for 20 min at 37°C to allow collagen polymerization.





**Figure 16.** The collagen assay, imaging DC migration in 3D. (A) Scheme of the setup and procedure to prepare a collagen assay. A cover glass is placed on top of the silicone drops and collagen mix. The edge of the cover glass should adhere to the silicone and the collagen/cell mixture should fill the entire cover glass. Avoid to add an excessive amount of collagen/cell mixture or to push the coverslip too strongly. On the other hand, prevent leaving empty spaces below the surface of the coverslip by adding a lower amount or by failing to push the coverslip. (B) Microscopic images (10× magnification, phase contrast) of bovine and (C) a rat collagen gel. Note that bovine collagen forms large bundle/fibers that might steer the DC migration during directional migration. Unlike bovine, rat collagen shows a much more homogeneous mixture. The inset on the right shows a microscopic image (40× magnification, phase contrast) the rat collagen fibers randomly organized, mimicking the extracellular matrix of a tissue. Calibration bars of 100 and 20  $\mu\text{m}$  are depicted.

14. Upon collagen polymerization, carefully add 2 ml of media. Add chemokines accordingly. Use >100 ng/ml CCL19 to analyze the chemotaxis, and >150 ng/ml CCL21 for the haptotaxis of DC. The suggestion is to use equimolar concentrations of both chemokines to have at least 10 nM.
15. To analyze dynamic cell behavior perform live cell imaging. To monitor cell migration in a large number of cells low magnification (10×) acquisition is enough (Fig. 16B and C). Use a system equipped with controlled atmosphere (5% CO<sub>2</sub> and 37°C). It is recommended to use phase contrast to better visualize the migration of cells, but bright field is also compatible. A frame rate of 2 min is enough to analyze overall DC migration. Faster imaging is also possible but it increases the file size and might limit the amount of positions.
16. For detailed analysis of DC shape during migration, 40× magnification and higher time frame acquisition (every 10 sec) are recommended (Fig. 16C).
17. Pause point. Once the collagen is polymerized and the cells are flooded with media, the samples could be kept for a few hours in the incubator until imaging.

## 7.4 Data analysis

The analysis of DC migration is performed using Fiji (Image J) software.

### 7.4.1 Analysis of DC migration in collagen (3D environments)

#### 7.4.1.1 image processing.

1. Rotate the movie so the cells migrate horizontally from left to right (according to the gradient). To found the right angle of rotation, highlight the migration of cells using Z-Projection (Stk - Z Project - Max Intensity). This will show a time projection of the trajectories and will help to define the rotation angle.
2. Crop the region of interest to  $x \mu\text{m}$  from the edge of the coverslip. This zone should be defined according to gradient shape and stability, but it is recommended to use a 250  $\mu\text{m}$  region close to the chemokine source.
3. To optimize the analysis of migration, it is recommended to first apply several filters as follows.
4. First create an average time projection of the movie (Stk - Z Projection - Average Intensity) and subtract it from the previously rotated movie (Process - Image Calculator). This step will remove the sessile cells from the analysis leaving only motile cells as white objects over a dark background.
5. Duplicate the processed movie and apply a Mean Filter of 20 (Process - Filters - Mean Filter). The radius should be adjusted depending on the cells used (DC: 20  $\mu\text{m}$ ). Subtract the gener-

ated image from the movie (as described in step 4), to remove light reflections from the cells.

6. Finally, apply a Gaussian Blur filter of 5  $\mu\text{m}$  (Process - Filters - Gaussian Blur). In order to visualize the cells in the processed images, adjust the brightness/contrast needs to be adjusted accordingly. The Gaussian filter causes a round white shape of the cells that can be easily tracked by the image software analysis.

#### 7.4.1.2 cell tracking.

1. For tracking migrating cells use TrackMate v7 plugin from Fiji (ImageJ) [107, 122]. Open the video created in 4.1.1 and start the tracking plugin (Plugins - Tracking - TrackMate).
2. In the first options panel adjust the spatiotemporal calibration of your data. This information is normally provided when reconstituting the movies using .nd files or it is given by the software during image acquisition. Click “next” to move to the following window.
3. In the next section, select a detection algorithm, offering a choice of 3: The Log detector is optimal for examining spot sizes between approximately 5 and 20 pixels in diameter (use this algorithm for DC). Use the DoG detector for small spot sizes less than approximately 5 pixels and the downsample LoG detector for spots larger than around 20 pixels in diameter.
4. Next, add an estimated blob diameter of 20 for DC. If required, enter a numerical value in the “Qualitative threshold”, although is preferable to avoid filtering object detection at this stage. Once optimal segmentation parameters are defined, the segmentation is started with the “next” button.
5. In the next window it is possible to adjust the performance, memory and hard disk usage. This step is only necessary for a very large number of spots (more than 1 million). For a smaller number of spots, just hit the “next” button.
6. In the next window named “Set filters on spots” the shape of violet circles with the previously defined diameter should appear. Irrelevant spots could be now removed. To do so, press the “+” button to add a feature filter. Select “Quality” filter. A small box should appear at the top containing a histogram. To move the threshold, click and drag in the histogram window. Choose 0.1 as filter value but adjust accordingly to your samples by verifying the right detection of spots in the image.
7. To create tracks with the selected spots, a compatible particle linking algorithm can be selected in the next window. Use the tracker “the nearest neighbor”. Here, each spot in one image is linked to another in the next image. You can set a maximum linking distance, which is indicated in the next window.
8. Add a maximum linking distance of 20 pixel, defined for the optimal diameter of an average DC spot. The log panel appears, in which the tracking process is shown.

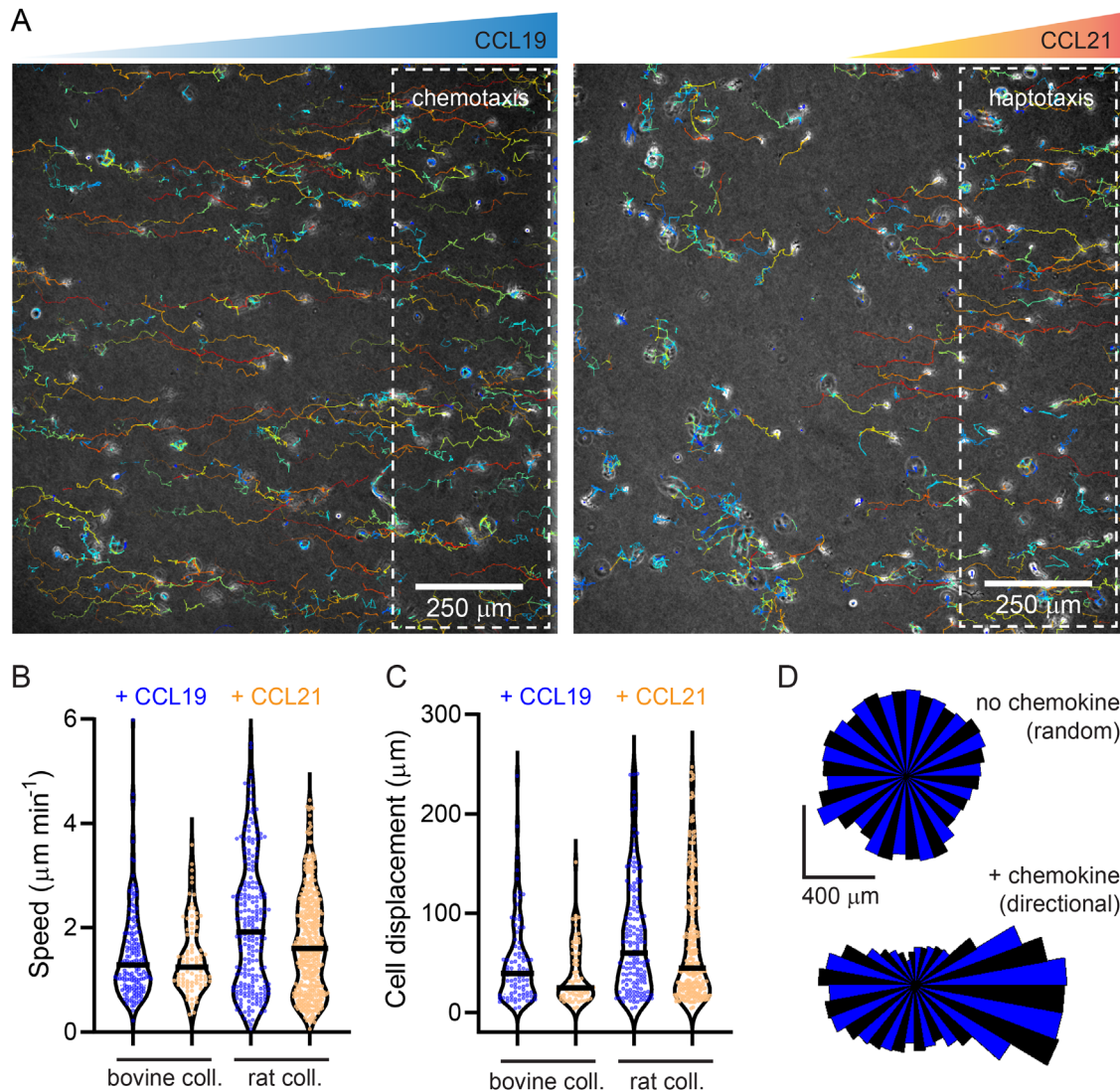
9. In the next dialogue box, the results of tracking will be displayed. Color-coded tracks will appear, showing the progression of the spots across the video. Here, tracks could be filtered according to the experimental parameters defined based of the sample’s behavior. For DC it is suggested to choose tracks longer than 10–20 min depending on the acquisition frame rate to have enough information about the migration of each cell. From the drop menu choose “Track duration” and adjust to 1200 or according to your sample.
10. In the last step the data is obtained. Click the button “Tracks” and export the sheets “Spots” and “Tracks” as CSV. These tables contain several parameters including: Track displacement, Track mean speed that could be plot in other software (Fig. 17A-C). A very useful tool is the color-coding of the tracks and their appearance along the movie of their full display (Fig. 17A). Once a visualization parameter is chosen the merged video containing the tracks and the cells can be exported in the next windows.
11. From the “Display Options” panel move “Next” two times until the panel “Select an action”. Choose “Capture overlay” from the drop menu and export the movie by choosing the first and last frame (Fig. 17A). It is also possible to export only the track by selecting “Hide image”.
12. For further information visit <https://imagej.net/plugins/trackmate/> and read the article by Ershov et al [122].

#### 7.4.1.3 Visualization of DC migration.

1. Use the chemotaxis and migration tool 2.0 from Ibidi to visualize the tracking data.
2. Open the table “Spots” obtained in 4.1.2 (step 10). Make a new table containing the data track ID, slice, x and y coordinates, or the data will not be uploaded. The first column should not start with the track ID. Remove dots and commas. Only the first row might contain the name of the parameter. Save the table as a .txt file.
3. Go to Migration tool 2.0 and “import data”, choose the .txt file from step 1.
4. Initialize the data under settings by specifying X/Y units and time intervals according to your imaging setup.
5. Once initialized, tracks can be plotted. Use the diagram plot “Rose diagram” (apply settings - diagrams - Plot “Rose diagram”) (Fig. 17D). This plot can be saved by right clicking on the diagram. Comparison between random and chemokine-induced migration is useful to evaluate changes in DC directional migration.
6. More information is contained in the Instructions of Chemotaxis and Migration Tool 2.0 (ibidi.com).

## 7.5 Pitfalls

1. After activation of the cells with LPS, the supernatant should carefully collected ensuring to keep the semi-adherent cells,



**Figure 17.** Analysis of DC migration in 3D. (A) Microscopy image of a field of view showing the migration tracks of DC undergoing chemotaxis in CCL19 gradient for 5 h. A region close to the chemokine source is highlighted with the dotted rectangle. A similar field of view obtained during DC haptotaxis towards CCL21. Note that on the left side of the field of view, DC migration remains random. (B) Graph showing the speed of migration induced by CCL19 and CCL21 in bovine or rat collagen (3 mg/ml). (C) Graph showing the cell displacement in the same conditions shown in (B).  $N = 3$  independent experiments,  $n = 500$  cells. (D) Rose plots depict the random migration of DC in absence of chemokine, and the directional migration towards the chemokine source (on the right side).

- and the flushing should be done gently to avoid recovering the adherent cells that do not migrate.
- Prepare the mixture with bovine collagen keeping everything on ice to prevent premature polymerization. Likewise, avoid desiccation of the collagen mixture, as this could decrease cell viability and/or change the diffusion of chemokines.
- Avoid the formation of bubbles during the preparation of the collagen mix, as these interfere with the migration of cells and distort the tracking analysis.
- Prevent freezing and thawing cycles of chemokines, because this could result in reduced effects in directional migration.

- If the chemokine will be added during the experiment acutely, prepare a stock of at least 100 μl in order to ensure proper diffusion and a quick effect during the duration of the experiment.
- Use very fine forceps to avoid breaking or damaging the glass cover, as this will result in the presence of debris during the microscopy.
- The Chemotaxis and Migration Tool 2.0 provides very useful analysis for directionality, which are otherwise not available in open access software. However, this software is not very user friendly, not intuitive, and the annotation of instructions is deficient.



## 8 Analyzing mouse skin DC migration by whole skin explant culture and FITC painting assays

### 8.1 Introduction

Dendritic cells (DC) are central players of the immune system as they initiate and modulate immune responses to invading pathogens. Therefore, DC are strategically positioned at the borders to the environment, like the skin, where they form a dense network to maintain cutaneous immune homeostasis [123, 124]. Upon pathogen/ antigen (Ag) recognition, DC mature from highly phagocytic cells into professional Ag-presenting cells along with their migration to tissue-draining LN, where they prime naïve T cells and initiate adaptive immune responses. Next to their crucial role in protective immunity, DC are also essential in maintaining immune tolerance towards self- and innocuous environmental Ag [125]. To fulfill these opposing tasks, the skin accommodates phenotypically and functionally specialized DC subtypes that can be distinguished by their differential expression of integrins, such as CD11b and CD103, as well as the C-type lectin receptor Langerin (CD207) [124].

Langerin<sup>+</sup> Langerhans cells (LC) are epidermis-resident Ag-presenting cells. Although LC are Flt3L-independent and, like many tissue-resident macrophages, of embryonic origin and self-maintain in situ, functionally they represent typical DC in that they migrate to draining lymph nodes (LN) and activate naïve Ag-specific T cells [126]. LC express cell-adhesion molecules such as Epcam (CD326) and E-cadherin, which allow them to form close cell contact with the surrounding keratinocytes [127]. Moreover, LC are the only APC population in the epidermis where they create the first line of defense against invading pathogens [128], but can also negatively regulate protective immunity during infection [129]. In contrast to the epidermis, the dermis harbors different subpopulations of dermal DC (dDC) comprising a small population of Langerin<sup>+</sup> DC and a major heterogeneous population of Langerin<sup>-</sup> CD11b<sup>low/+</sup> DC [130]. Langerin<sup>+</sup> conventional DC (cDC)1, identified by the expression of the chemokine receptor XCR1 [131], can be further subdivided into CD103<sup>+</sup> and CD103<sup>-</sup> subsets, which both display low expression of CD11b and Epcam [130]. These Langerin<sup>+</sup> cDC1 are critical to cross-present exogenous Ag to CD8<sup>+</sup> T cells promoting cytotoxicity [132], however, cross-presentation capacity was also demonstrated for Langerin<sup>-</sup> CD11b<sup>low</sup> cDC2 [133] and LC [134]. Langerin<sup>-</sup> CD11b<sup>+</sup> cDC2 are also competent to prime cytotoxic T cells [135] as well as efficient in presenting antigens on MHC class II to CD4<sup>+</sup> T cells [136].

Mobilization and migration are critical features of effective LC and DC function and therefore important to study. Here, we describe two complementary protocols for the analysis of skin DC migration: firstly, an *ex vivo* DC migration assay of mouse ear skin explants (the so-called ‘crawl-out assay’) and secondly, FITC-painting as an *in vivo* assay to monitor DC trafficking to skin-draining LN in an inflammatory setting. Moreover, for both methods, we provide an easy and ready-to-use protocol for the flow

cytometric analysis of the distinct cutaneous DC subtypes using a conserved core marker antibody panel.

**Table 22.** General reagents for crawl-out and FITC assay

Reagent	Manufacturer	Catalogue number
RPMI-1640 + L-Glutamin	Gibco	21870076
Fetal Bovine Serum (FCS)	Sigma	F7524
Phosphate Buffered Saline (PBS) without calcium and magnesium	Sigma	D8537
Ethylendiamintetraacetat (EDTA) (0.5M)	Sigma	E5134-500G
FC Block (Anti-CD16/32, Anti-FcγRIIB/777)	BioxCel	BE0307
Cytofix/Cxtoperm	BD	51-2090 KZ
Perm/Wash buffer	BD	51-2091 KZ
Trypan blue	Gibco	15250-061

**Table 23.** General solutions for the crawl-out and FITC assays

Solution	Ingredients	End concentration
PBS/ EDTA solution	PBS	
FACS buffer	EDTA	2 mM EDTA
	PBS	
	EDTA	2 mM EDTA
	FCS	3 %

**Table 24.** Specific reagents for the crawl out assay

Reagent	Manufacturer	Catalogue number
Penicillin/Streptomycin	Gibco	15140-122

### 8.2 Materials

#### 8.2.1 General reagents required for the crawl-out and FITC painting assays

All necessary reagents that are needed for the crawl-out as well as for the FITC painting assay are listed in Table 22.

#### 8.2.2 General solutions for the crawl-out and FITC painting assays

All necessary solutions that are needed for the crawl-out as well as for the FITC painting assay are listed in Table 23.

#### 8.2.3 Reagents specific for the crawl-out assay

All necessary reagents that are specifically needed for the crawl-out assay are listed in Table 24.



**Table 25.** Specific solutions for the crawl-out assay

Solution	Ingredients	End concentration
R10-medium	RPMI (+ glutamine)	10%
	FCS	100 U/mL
	Penicillin/Streptomycin	

**Table 26.** Specific reagents for the FITC painting assay

Reagent	Manufacturer	Catalogue number
DMSO	Sigma	67-68-5
Dibutyl phalate (DBP)	Sigma	D2270
Aceton	ApliChem	131007.1211
FITC (Fluorescence isothiocyanate isomer I)	Sigma	F7250
Collagenase IV	Worthington	LS0004186
Deoxyribonuclease I (DNaseI)	Roche	10104159001

**Table 27.** Specific solutions for the FITC painting assay

Solution	Ingredients	End concentration
LN Digestion mix	RPMI (+ glutamine)	
	Collagenase IV	200 U/mL
	DNase I	0.5 U/mL

### 8.2.4 Solutions specific for the crawl-out assay

All necessary solutions that are specifically needed for the crawl-out assay are listed in Table 25.

### 8.2.5 Reagents specific for the FITC painting assay

All necessary reagents that are specifically needed for the FITC painting assay are listed in Table 26.

### 8.2.6 Solutions specific for the FITC painting assay

All necessary solutions that are specifically needed for the FITC painting assay are listed in Table 27.

### 8.2.7 General equipment

All necessary equipment that is needed for the crawl-out as well as for the FITC painting assay is listed in Table 28.

### 8.2.8 Equipment specific for the crawl-out assay

All necessary equipment that is specifically needed for the crawl-out assay is listed in Table 29.

**Table 28.** General equipment for the crawl-out and the FITC painting assay

Equipment	Manufacturer	Catalogue number
Forceps & Scissors	Sharp tissue scissors HSB Hammacher	HSB-390-10 (51807020)
Falcon (50mL)	Greiner bio-one	227261
96-well plate (V shape)	Thermo Scientific	163320
1.5 mL tubes	Sarstedt AG	72.690.001
70 um cell strainer	Falcon	10082019
Neubauer chamber	Superior Marienfeld	
Microscope	Olympus CK2	
Centrifuge "Z 446 K"	Hermle Labor Technik	
PipetBoy	Fisher Scientific	
FACS "Canto II"	BD	

**Table 29.** Special equipment for the crawl-out assay

Equipment	Manufacturer	Catalogue number
Incubator (37°C, 5% CO <sub>2</sub> )	Heraeus	BBD6220
24-well plate	Thermo Fisher Scientific Inc.	160007

**Table 30.** Special equipment for the FITC painting assay

Equipment	Manufacturer	Catalogue number
Isoflurane Vaporizer	UniVet Porta	
Thermo shaker	Laboratory Technology Buddeberg	

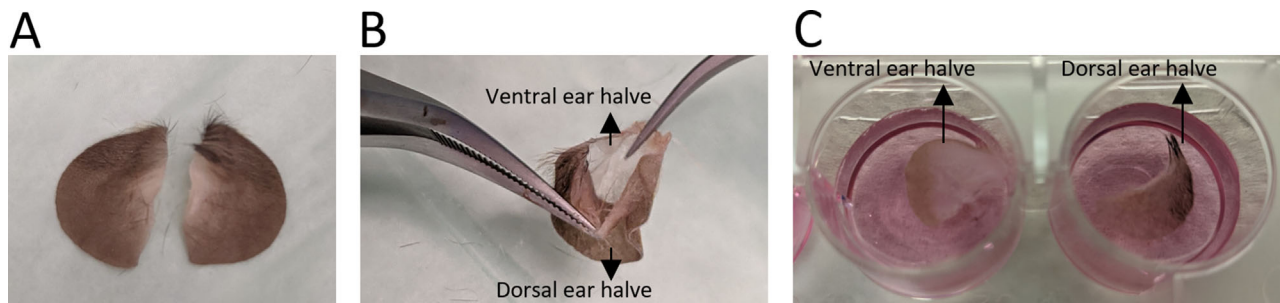
### 8.2.9 Equipment specific for the FITC painting assay

All necessary equipment that is specifically needed for the FITC painting assay is listed in Table 30.

## 8.3 Step-by-step sample preparation for the crawl-out assay

### 8.3.1 Preparation of ear tissue

1. Cut off the ears at the base of the mouse ears (Fig. 18A)
2. Split the ears into dorsal and ventral halves with strong forceps (Fig. 18B)
3. Add 2mL of R10-medium into a 24-well plate
4. Place each of the four ear halves with the dermal side down onto medium in a 24-well plate (one ear per well, skin should float on medium) (Fig. 18C)
5. Culture ear skin for 24h or 48h in the incubator (37°C, 5% CO<sub>2</sub>) to allow skin DC to emigrate from tissue



**Figure 18.** Illustration of the different steps of the crawl-out assay. Ears are cut off at the base of the mouse ear cartilage (A) and afterward split with two fine forceps to separate ventral and dorsal sides from each other (B). Subsequently, each ear halve is placed with the dermal side down into a separate well (24-well plate) filled with 2 mL medium (C).

### 8.3.2 Harvesting cells after 24h and 48h

All following steps can be performed under non-sterile conditions.

- Put the 24-well plate containing ear skin explants on ice for 10 min.
- Remove skin from medium by pulling the dermal side over the well edge to catch migratory DC stuck to the dermal side and harvest the cells by transferring the culture medium into a 50 mL Falcon tube
- Wash each well by adding 2 mL PBS/EDTA solution and incubate the plate for 10 min. in the incubator (37°C, 5% CO<sub>2</sub>)
- Collect leftover cells and add them into the 50 mL Falcon tube
- Centrifuge cells for 5 min. at 450 × g at 4°C
- Resuspend the pellet in 500 μL of PBS/EDTA solution and count cells

## 8.4 Step-by-step sample preparation for the FITC painting assay

### 8.4.1 Applying FITC reagent mix on mouse ear skin

- Dissolve FITC powder in DMSO (Table 31)
- Mix Aceton with DBP at a ratio of 1:1
- Dilute FITC dissolved in DMSO 1:10 in Aceton/DBP and mix well

**Table 31.** List of different concentrations that can be used for the FITC painting assay

% FITC	FITC per 100 μL DMSO
1%	1 mg
5%	5 mg
7.5%	7.5 mg
10%	10 mg

Note: FITC concentrations vary in the literature and should be determined on an individual basis; most commonly used concentrations range from 1–5 %

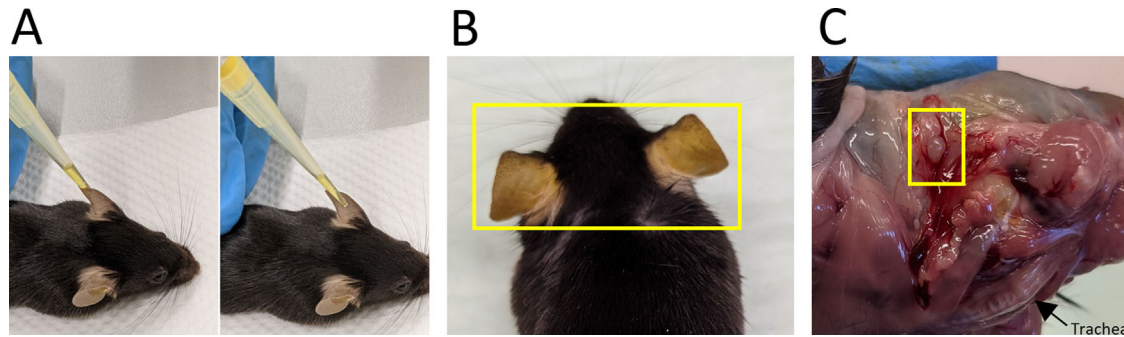
- Anaesthetize mice with Isoflurane (0.5 L/min. O<sub>2</sub>, 3 L/min. Isoflurane)
- Paint both ears with 25 μL FITC solution per ear (dorsal and ventral side) (Fig. 19A&B)
- Keep mice anesthetized until FITC solution has dried on both ears
- Isolate the skin-draining cervical LN after 24h and 48h (Fig. 19C)

**Note:** Isolate skin-draining inguinal LN not draining the FITC-painted site to serve as a negative control

### 8.4.2 Single-cell suspension of lymph nodes

All following steps can be performed under non-sterile conditions.

- Put both cervical LN in a 1.5 mL Eppendorf tube filled with 10 μL PBS and check that both LN are covered in liquid (so that they will not dry out)
- Add about 200 μL of LN digestion mix
- Cut the LN into very small pieces with a fine scissor
- Add another 800 μL of digestion mix and shake the samples at 1200 rpm for 45 min. at 37°C
- Add 20 μL of 500 mM EDTA solution per 1 mL digestion mix (final concentration of EDTA in 1mL is 10 mM) for 5 min. at room temperature
- Vortex cell suspension and pass it over a 70 μM cell strainer
- Wash the 1.5 mL Eppendorf tube with 1 mL PBS/EDTA solution and also pass it over the 70 μM cell strainer
- Wash the cell strainer with 10 mL PBS/EDTA solution
- Centrifuge cells for 5 min. at 450 × g at 4°C
- Resuspend the pellet in 1 mL PBS/EDTA solution and count cells
- Centrifuge cells for 5 min. at 450 × g at 4°C
- Resuspend the cell pellet in a volume of FACS buffer to reach 2 × 10<sup>6</sup> cells/ 100 μL



**Figure 19.** Experimental steps during FITC painting. Mice are anesthetized with Isoflurane (0.5 L/min O<sub>2</sub>, 3 L/min Isoflurane) and 25  $\mu$ L of FITC solution is equally distributed on the dorsal and ventral side of each ear (A). After 24h and 48h of FITC painting, ears of mice appear slightly yellow (B). At these time points, the cervical lymph nodes are swollen and also appear slightly yellow (for anatomical orientation the trachea is indicated with a black arrow) (C).

**Table 32.** List of surface staining antibodies including the used fluorochrome, company, clone, and dilution

Fluorochrome	Surface molecule	Company	Clone	Dilution
FITC*	CD24 (optional)	Biolegend	M1/69	1:200
APC-Cy7	CD11c	Biolegend	N418	1:200
PE	CD11b	eBioscience	M1/70	1:1000
PE-Cy7	CD326 (Epcam)	eBioscience	G8.8	1:800
ef506	Fixation Viability dyes	eBioscience	–	1:1000
PerCp-Cy 5.5	CD103	Biolegend	2E7	1:100
ef450	MHCII (I-A/I-E)	eBioscience	M5/114.15.2	1:4000

\* Note for FITC painting: Leave the FITC channel empty without any staining antibody to detect the FITC<sup>+</sup> DC that have migrated to the LN

## 8.5 General flow cytometry staining

All following steps can be performed under non-sterile conditions.

1. Take all the cells from one mouse (from four ear halves) for one flow cytometry staining
2. Pipet cells into a V-bottom 96-well plate
3. Commercially available, purified rat anti-mouse anti-CD16/32 is employed to block the Fc-gamma RIIB/III receptors, thereby avoiding unspecific recognition of staining antibodies by surface Fc-gamma receptors. For this, add 10  $\mu$ L of Fc solution to the cells and incubate for 15 min. at room temperature and centrifuge cells for 5 min. at 450  $\times$  g at 4°C
4. Resuspend cells in 50  $\mu$ L cell surface marker antibody master mix (Table 32) in FACS buffer and incubate in the dark at 4°C for at least 20 min.
5. Add 100  $\mu$ L FACS buffer to each well to stop the staining
6. Centrifuge cells for 5 min. at 450  $\times$  g at 4°C
7. Add again 100  $\mu$ L FACS buffer and resuspend the cell pellet
8. Centrifuge cells again for 5 min. at 450  $\times$  g at 4°C
9. Fix the cells by resuspending the cell pellet in 100  $\mu$ L of BD Cyofix/Cytoperm solution for 45 min. in the dark at room temperature
10. Add 100  $\mu$ L of 1:10 diluted (in H<sub>2</sub>O) BD Perm/Wash buffer and centrifuge for 5 min. at 450  $\times$  g at 4°C
11. Stain for intracellular molecules or molecules that need to be stained with a preceding membrane permeabilization step to improve staining intensity (here: Langerin, CD207) in 50  $\mu$ L BD Perm/Wash buffer for at least 30 min. or overnight in the dark at 4°C (Table 33)
12. Add 100  $\mu$ L BD Perm/Wash buffer and centrifuge for 5 min. at 450  $\times$  g at 4°C
13. For washing the cells resuspend the cell pellet in 100  $\mu$ L BD Perm/Wash buffer and centrifuge for 5 min. at 450  $\times$  g at 4°C
14. Wash the cells again with 100  $\mu$ L FACS buffer and centrifuge for 5 min. at 450  $\times$  g at 4°C
15. Resuspend the cell pellet in 100  $\mu$ L FACS buffer
16. Cells are ready for flow cytometry analysis

## 8.6 Data analysis & gating strategy of migrated ear DC

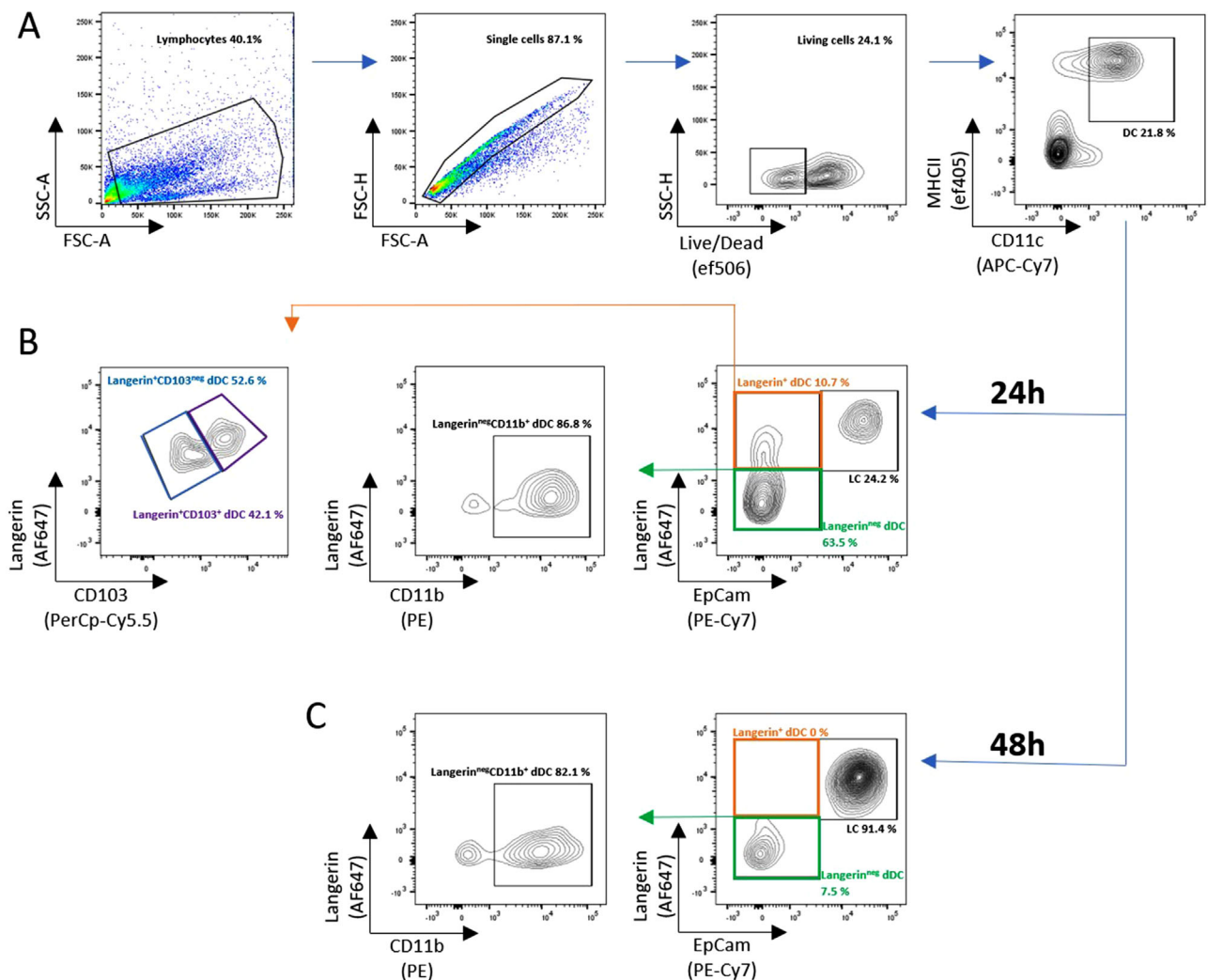
Data acquisition of the samples was performed at a BD Canto II analyzer with a blue (488 nm), a violet (405 nm), and red (633 nm) laser. Possible channels (and their filters) that can be used are: FITC (530/30 nm), PE (564/42 nm), PerCp (>670 nm), PeCy7 (780/60 nm), APC (660/20 nm), APC-Cy7 (780/60), Pacific blue (450/40), and Amcyan (530/70 nm). Data were analyzed by FlowJo software (V10.8.0). In the following, an example of a gating strategy for crawl-out DC (24h and 48h, Fig. 20) and FITC<sup>+</sup> LN DC (24h and 48h, Fig. 21) is provided:

**Table 33.** List of molecules that need to be stained after membrane permeabilization including the used fluorochrome, company, clone and dilution

Fluorochrome	Surface molecule	Company	Clone	Dilution
AF647	CD207 (Langerin)	Dendritics	929F3.01	1:200

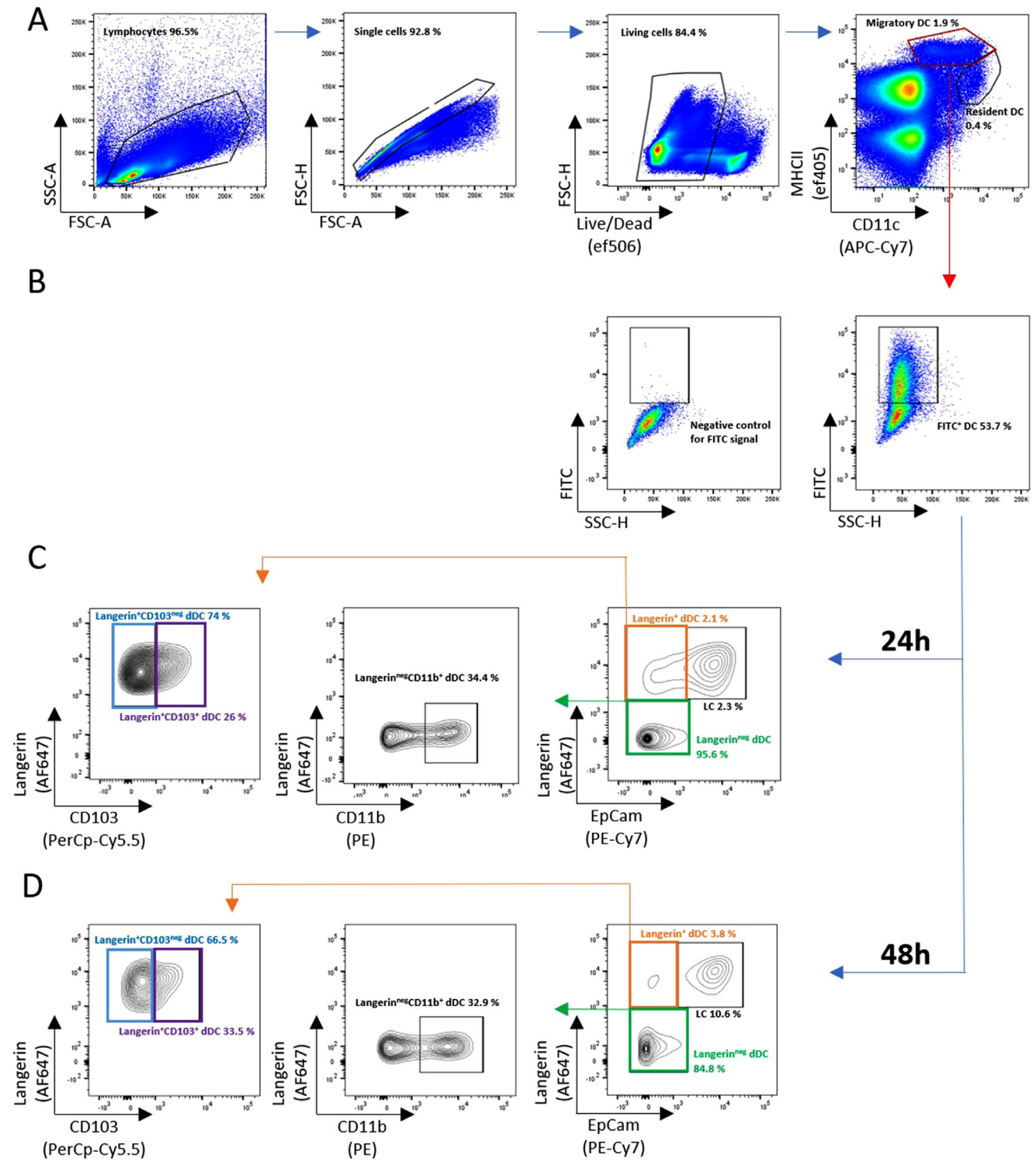
### 8.7 Pitfalls and top tricks

- Crawl-out assay:** It is possible to digest the ear skin to characterize the 'left over' DC in the skin after 24h or 48h (digestion protocol for ears see section 1.1. of **Guidelines for DC preparation and flow cytometry analysis of mouse non-lymphoid tissues** [137]). The skin is a non-lymphoid tissue and harbors not only leukocytes. Therefore, it is recommended to identify
- immune cells by using a leukocyte marker, such as the leukocyte common antigen CD45.**
- FITC-painting:** Always take a skin-draining LN as a negative control to facilitate the setting of the right gate for the FITC-signal during flow cytometry analysis. This can be either an ear skin-draining LN from an untreated mouse or a skin-draining LN of a FITC-treated mouse distant from the ears (e.g. an inguinal LN).



**Figure 20.** Example gating strategy for flow cytometry analysis of crawl-out DC after 24h and 48h incubation. Cells emigrated from ear skin explants were stained and first analyzed by FSC-A and SSC-A to exclude debris, followed by elimination of doublets. DC are identified as living MHCII<sup>+</sup>CD11c<sup>+</sup> cells (A), and gated into the different DC subsets at 24 h (B) and 48 h (C) of explant culture. Langerhans cells (LC) and dermal DC (dDC) subsets were categorized by their expression of Langerin and EpCam. LC are double-positive for EpCam and Langerin, Langerin<sup>+</sup> dDC are only positive for Langerin and Langerin<sup>-</sup> dDC are negative for both markers. Langerin<sup>+</sup> dDC were further divided into CD103<sup>-</sup> and CD103<sup>+</sup> subsets. Furthermore, CD11b<sup>+</sup> dDC are identified as CD11b<sup>+</sup>Langerin<sup>-</sup> EpCam<sup>-</sup> DC.





**Figure 21.** Example gating strategy for flow cytometry analysis of FITC<sup>+</sup> DC 24h and 48h after FITC painting. Single cells of skin-draining LN were stained and analyzed by FSC-A and SSC-A to exclude debris, followed by elimination of doublets. DC are identified as living MHCII<sup>+</sup> CD11c<sup>+</sup> cells. Among these cells, migratory DC can be distinguished from their LN-resident counterparts by higher expression of MHCII and lower expression of CD11c (A). The frequency of FITC<sup>+</sup> DC within the migratory DC population was determined by gating on a FITC-negative control (see Notes below) (B). Subsequently, migratory FITC<sup>+</sup> DC were divided into the distinct subsets (C) and 48h (D) after FITC painting. Langerhans cells (LC) and dermal DC (dDC) subsets were categorized by their expression of Langerin and EpCam. LC are double-positive for EpCam and Langerin, Langerin<sup>+</sup> dDC are only positive for Langerin and Langerin<sup>-</sup> dDC are negative for both markers. Langerin<sup>+</sup> dDC were further divided into CD103<sup>-</sup> and CD103<sup>+</sup> subsets. Furthermore, CD11b<sup>+</sup> dDC are identified as CD11b<sup>+</sup> Langerin<sup>-</sup> EpCam<sup>-</sup> DC.

- **Induction of TSLP secretion after FITC painting:** DBP in the FITC-solution triggers thymic stromal lymphopoietin (TSLP) secretion by keratinocytes and therefore this assay represents an example of Th2-driven inflammation [138].
- **TRITC as an alternative for FITC:** Using FITC as a cell tracker for the observation of *in vivo* skin DC migration can be critical when working with transgenic GFP reporter mice. In this case, TRITC (red) can be used instead of FITC (green) to monitor sdLN migration [139].
- **Staining of CD24 in skin and skin-draining LN:** In the skin, it is possible to use CD24 instead of Langerin (CD207) to distinguish Langerin<sup>+</sup> dermal DC as well as LC. This has the advantage that only surface staining is needed and cells do not need to be permeabilized. Notably, the expression of CD24 is upregulated on DC during migration, thus it is not advisable to use CD24 in LN as a discriminating marker for CD24<sup>high</sup> DC (which are also Langerin-positive) and CD24<sup>low</sup> DC (which are Langerin-negative and do not just represent migrated DC) [130].
- **Migration of LC:** Once LC have been activated, they downregulate EpCam expression to gain mobility and emigrate from the epidermis [140]. Special about LC is that their migration to the skin-draining LN occurs in two steps: first, LC migrate from the epidermis to the dermis across the basement membrane at the dermal-epidermal junction and then they traffic from the dermis to the draining LN with the afferent lymph [141].
- **Kinetics of DC subset migration:** In contrast to dermal DC, LC need to pass the basement membrane at the dermal-epidermal border to reach the draining lymphatics. Therefore, dermal DC and LC display different migration kinetics especially detectable in the crawl-out assay. While the majority of dermal DC have emigrated 24h after activation, LC are the main population of migratory DC appearing at 48h (Fig. 20).

## 9 Measuring inflammasome activity in human primary DC

### 9.1 Introduction

Inflammasomes are multiprotein complexes in the cytosol that are crucial for the activation of caspase-1 in order to cleave pro-IL-1 $\beta$  and pro-IL-18 into their mature form [142, 143]. Further, caspase-1 cleaves the protein gasdermin D leading to the insertion of gasdermin D into the cell membrane [144–146]. Thereby, gasdermin D forms pores enabling the diffusion of IL-1 $\beta$  and IL-18 into the extracellular space as well as inducing an inflammatory form of cell death called pyroptosis. In human and mice, several different inflammasomes exist, which mainly differ in the sensor protein used for detecting danger and pathogen-associated signals [142, 143]. They however all use the protein apoptosis-associated speck-like protein containing a CARD (ASC) for multimerization and recruitment of pro-caspase-1, which subsequently activates itself by autoproteolysis. The best-studied inflammasome

is the NLR family pyrin domain containing 3 (NLRP3) inflammasome, which recognizes a diverse set of ligands including danger-associated molecular patterns derived from host cells (e.g., intracellular stress and damage) as well as pathogen-associated molecular patterns deriving from bacteria or viruses [142, 147]. Besides their broad proinflammatory functions, both IL-18 as well as IL-1 $\beta$  are important for the polarization of CD4<sup>+</sup> T cells into Th1 and Th17 cells, respectively [148–150]. Thereby, activation of the inflammasome might contribute to protective immune responses against invading pathogens [142, 151]. However, overt activation of the inflammasome is associated with different chronic inflammatory diseases such as rheumatoid arthritis [152].

The capacity of DC to activate the inflammasome and secrete IL-1 $\beta$  is controversially discussed [142]. Most of the data were generated using bone marrow- (mouse) or monocyte-derived (human) DC, while only few groups used bona fide primary DC: here, it is clearly shown that human primary cDC2 are able to activate the NLRP3 inflammasome, while it seems that in mouse DC activation is partially inhibited [142, 153–155]. In this section, we provide a protocol to measure inflammasome activity on different level (i.e., secretion of inflammasome-dependent cytokines, inflammasome-induced cell death (pyroptosis), activation of the effector caspase-1) for cell sorted primary human DC [153].

### 9.2 Materials

#### 9.2.1 Reagents

A complete list of reagents is provided in Table 34. Further, cell-sorted primary DC are needed (see [156] section 3 “Cell sorting of primary human DC”).

#### 9.2.2 Equipment

Necessary equipment is listed in Table 35.

### 9.3 Step-by-step sample preparation

#### 9.3.1 Preparation of stocks and solutions

##### DC medium

Add 5% human sera type AB (v/v), 5% Panexin NTS (v/v), 5% Panexin NTA (v/v), 1% Penicillin-Streptomycin (v/v), 1% L-Glutamine (v/v), and 1% sodium pyruvate (v/v) to 500 ml RPMI-1640.

##### ATP

Prepare a 200 mM stock solution of ATP by adding 9.072 ml of endotoxin-free water to 1 g of ATP. Vortex the solution until it is completely dissolved. Prepare aliquots and store them at  $-20^{\circ}\text{C}$ .

**Table 34.** List of needed reagents for the preparation of single cells from human lymphoid tissues

Reagent	Manufacturer	Ordering Number
Chemicals & Solutions		
Dulbecco's Phosphate Buffered Saline without calcium and magnesium	Sigma-Aldrich	D8537
RPMI-1640 Medium	Sigma-Aldrich	R8758
Human Serum type AB (male)	Sigma-Aldrich	H4522
Trypan blue	Sigma-Aldrich	T6146
Adenosine 5'-triphosphate disodium salt (ATP)	Invivogen	tlrl-atpl
R848 (Resiquimod)	Invivogen	tlrl-r848
4',6-diamidino-2-phenylindole (DAPI)	Thermo Scientific	62247
Panexin NTS	PAN Biotech	P04-95080P
Panexin NTA	PAN Biotech	P04-95070
Penicillin-Streptomycin	Sigma-Aldrich	P4333
L-Glutamine	Sigma-Aldrich	G7513
Sodium Pyruvate Kits	Sigma-Aldrich	S8636
LDH-Cytox™ Assay Kit	BioLegend	426401
FAM FLICA™ Caspase-1 Kit	Bio-Rad	ICT097
LEGENDplex Human Cytokine 2 Panel	BioLegend	740102

**R848**

Prepare a 1 mg/ml stock solution of R848 by adding 500 µl of endotoxin-free water to 500 µg of R848. Vortex the solution until it is completely dissolved. Prepare aliquots and store them at -20°C.

**1 × Wash buffer (LEGENDplex)**

Dilute 2 ml of 20 × Wash buffer with 38 ml of H<sub>2</sub>O.

**Table 35.** Necessary equipment

Equipment	Company	Purpose
Centrifuge "Allegra X-15R"	Beckman-Coulter	Centrifugation of 50 ml tubes, 15 ml tubes, and V-bottom plates
Incubator "HERAEUS BBD6220"	Thermo Scientific	Cabinet-style incubator with 5% CO <sub>2</sub> and 96% relative humidity for the lymphoid tissue digestion
LSR Fortessa (#647800)	BD	Flow cytometric analysis of single-cell suspensions
Neubauer chamber 0.100 mm; 0.0025 mm <sup>2</sup>	Superior Marienfeld	Cell counting
Sterile bench "Mars Safety Class 2"	Scanlaf	Performance of all aseptic procedures
96 Well Cell Culture Plate (V-bottom) (#65180)	Greiner bio-one	Plates for the stimulation of sorted DC in sterile conditions
Clear Flat-Bottom Immuno Non-sterile 96-Well Plates (#442404)	Thermo Scientific	Performance of the LDH assay
50 ml tubes (#352070)	Falcon	Preparation of 1 × Wash buffer (LEGENDplex assay)
Serological pipettes (#606180)	Greiner bio-one	Pipetting
VersaMax enzyme-linked immunosorbent assay plate reader	Molecular Devices	Measuring of absorbance at 490 nm to detect cell lysis

**Working solution for LDH-cytox assay**

To prepare the Working Solution, add 5 ml volume of Assay Buffer to the Dye Mixture vial. Close the cap and dissolve the contents completely. Add back the whole volume of the mixture prepared to the Assay Buffer bottle. Store the Working Solution at 4°C and protected from light. The working solution is stable for up to 6 months after mixing.

**FAM FLICA**

Prepare a 150x stock of FAM FLICA by reconstituting a vial of FAM FLICA in 50 µl DMSO. Aliquot the stock and store it up to six months at -20°C.

**9.3.2 Stimulation of the NLRP3 inflammasome in human DC with ATP****9.3.2.1 Measuring of secreted IL-1β.****9.3.2.1.1 Stimulation of the NLRP3 inflammasome.**

1. Isolate primary human cDC1, cDC2, pDC, and monocytes as described in section 3 "Cell sorting of primary human DC" in [156]. Resuspend the isolated DC in DC medium to a final concentration of  $4 \times 10^5$  cells/ml.
2. Prepare a 10 µg/ml solution of R848 by diluting R848 stock solution 1:100 in DC medium. Dilute the 200 mM stock solution of ATP 1:40 in DC medium to reach a final concentration of 5 mM.
3. Transfer  $4 \times 10^4$  DC or monocytes (100 µl of the prepared suspension in step 1) into a well of a sterile 96-well V-bottom plate. For each cell population to be analyzed, prepare four wells (see Table 36).
4. Mix two wells of the cells for each cell population 1:1 with DC medium (control without priming), while the other two wells for each cell population are diluted 1:1 with diluted R848

**Table 36.** Exemplary scheme for inflammasome stimulation for measuring of secreted IL-1 $\beta$  (9.3.2.1.)

Cell population	Stimulation	Well 1	Well 2	Well 3	Well 4
cDC1	1 <sup>st</sup> (priming)	DC medium	DC medium	5 $\mu$ g/ml R848	5 $\mu$ g/ml R848
	2 <sup>nd</sup> (Inflam.)	DC medium	5 mM ATP	DC medium	5 mM ATP
cDC2	1 <sup>st</sup> (priming)	DC medium	DC medium	5 $\mu$ g/ml R848	5 $\mu$ g/ml R848
	2 <sup>nd</sup> (Inflam.)	DC medium	5 mM ATP	DC medium	5 mM ATP
pDC	1 <sup>st</sup> (priming)	DC medium	DC medium	5 $\mu$ g/ml R848	5 $\mu$ g/ml R848
	2 <sup>nd</sup> (Inflam.)	DC medium	5 mM ATP	DC medium	5 mM ATP
Monocytes	1 <sup>st</sup> (priming)	DC medium	DC medium	5 $\mu$ g/ml R848	5 $\mu$ g/ml R848
	2 <sup>nd</sup> (Inflam.)	DC medium	5 mM ATP	DC medium	5 mM ATP

Inflam. = Inflammasome ligand

**Table 37.** Scheme for serial dilutions for the standard curve to measure secreted IL-1  $\beta$  (9.3.2.1.)

	C7	C6	C5	C4	C3	C2	C1	C0
Dilution	Undiluted	1:4	1:16	1:64	1:256	1:1024	1:4096	blank

solution to reach a final concentration of 5  $\mu$ g/ml R848 (see Table 36). Incubate the cells at 37°C for 3 h.

- Heat 10 ml of DC medium to 37°C. Preheat the prepared 5 mM ATP solution.
- After the 3 h incubation step, centrifuge the plate for 10 min with 520  $\times$  g at RT. With a pipette, remove as much liquid as possible without touching the cell pellet. Wash the cells with 100  $\mu$ l of pre-warmed DC medium. Centrifuge the plate for 10 min with 520  $\times$  g at RT.
- Remove the supernatant with a pipette and add 200  $\mu$ l of prewarmed DC medium to one of the wells cultured in DC medium and one of the wells treated with R848. To the remaining wells (one cultured in DC medium and one treated with R848) add 200  $\mu$ l of the diluted ATP solution (see Table 36).
- Resuspend the cells and incubate for 3–12 h at 37°C.
- After the incubation time, centrifuge the plate for 10 min with 520  $\times$  g at 4°C. Harvest the supernatants using a pipette and store either at –80°C or directly perform cytometric bead array or ELISA to measure concentration of secreted cytokines (see section 9.3.2.1.2.).

#### 9.3.2.1.2 Measuring cytokines in the supernatant of the cells.

- If supernatants were stored at –80°C, thaw the supernatants. Warm up all contents of the LEGENDplex Human Cytokine Panel 2 to RT before starting with the assay. Prepare a stock solution of the lyophilized cytokines for the standard curve by adding 250  $\mu$ l of the assay buffer to the glass vial. Incubate it at RT for at least 15 min. Then, slightly pipette up and down. Prepare serial dilutions (1:4) by mixing 25  $\mu$ l of the standard with 75  $\mu$ l of assay buffer. Repeat this step five times by using the diluted standard for the next dilution. In the end, one should have the top standard (C7) as well as six dilutions as shown in the scheme below (Table 37).

- Pipette 25  $\mu$ l of assay buffer to 16 wells of a 96-well V-bottom plate for the standard curve (duplicates of each dilution and two blanks). Further, add 25  $\mu$ l of assay buffer to a well for each sample that will be analyzed. For the scheme shown above (Table 36), we would need 16 additional wells.
- Add 25  $\mu$ l of the C1–C7 to the standard wells (duplicates). Add 25  $\mu$ l of assay buffer to the remaining standard wells (C0 = blank; as well in duplicates). Add 25  $\mu$ l of the supernatants of the DC subpopulations and monocytes to the corresponding wells on the 96-well plate.
- Vortex the capture beads vial for at least 30 s. Then, add 25  $\mu$ l of the capture beads to each well of the standard curve (C0–C7) and the samples. Seal the plate with a provided plate sealer and incubate on a plate shaker (800 rpm) in the dark for 2 h.
- Centrifuge the plate with 250  $\times$  g for 5 min at RT. Dump the supernatant into a sink and blot the plate on a stack of clean paper towel. Alternatively, remove as much liquid as possible with a pipette.
- Wash the plate by dispensing 200  $\mu$ l of 1  $\times$  Wash Buffer into each well and incubate for one minute. Repeat step 14.
- Add 25  $\mu$ l of Detection Antibodies to each well. Resuspend the beads, seal the plate with a provided plate sealer and incubate the plate on a plate shaker (800 rpm) in the dark for 1 h.
- Without washing, add 25  $\mu$ l of Streptavidin-PE to each well. Seal the plate with a provided plate sealer and incubate the plate on a plate shaker (800 rpm) in the dark for 30 min
- Add 100  $\mu$ l 1  $\times$  Wash Buffer to each well. Repeat steps 14 and 15.
- Add 150  $\mu$ l of 1  $\times$  Wash Buffer to each well and acquire the samples on a flow cytometer. Cytometer setup can be performed using the cytometer setup beads provided by the set. Follow the instructions in the manual of the manufacturer. Briefly, record 300 events per bead. As we measure 13 cytokines, 3,900 events are recorded. Set the record/storage



**Table 38.** Exemplary scheme for inflammasome stimulation for measuring of pyroptosis (9.3.2.2.),

Cells	Well 1 high control	Well 2 low control	Well 3	Well 4	Well 5	Well 6
cDC1	DC medium	DC medium	DC medium	DC medium	5 µg/ml R848	5 µg/ml R848
	DC medium	DC medium	DC medium	5 mM ATP	DC medium	5 mM ATP
cDC2	DC medium	DC medium	DC medium	DC medium	5 µg/ml R848	5 µg/ml R848
	DC medium	DC medium	DC medium	5 mM ATP	DC medium	5 mM ATP
pDC	DC medium	DC medium	DC medium	DC medium	5 µg/ml R848	5 µg/ml R848
	DC medium	DC medium	DC medium	5 mM ATP	DC medium	5 mM ATP
Monocytes	DC medium	DC medium	DC medium	DC medium	5 µg/ml R848	5 µg/ml R848
	DC medium	DC medium	DC medium	5 mM ATP	DC medium	5 mM ATP

gate to the bead population containing beads A and B so that only the beads are recorded and saved. This will simplify the analysis using the LEGENDplex Software suite (see section 9.4.1). Adjust laser voltages for FITC, APC, and PE according to the manual and dependent on the used flow cytometer (for a LSRFortessa as used in this section, only APC and PE are needed). After cytometer setup, begin with sample acquisition with the standard curve (C0-C7) starting with the lowest concentration (C0).

### 9.3.2.2 Measuring of inflammasome-induced pyroptosis.

- Isolate primary human cDC1, cDC2, pDC, and monocytes as described in section 3 “Cell sorting of primary human DC” in [156]. Resuspend the isolated DC in DC medium to a final concentration of  $2 \times 10^6$  cells/ml.
- Transfer  $1 \times 10^5$  DC or monocytes (50 µl of the prepared suspension in step 20) into a well of a sterile 96-well V-bottom plate. For each cell population, at least six wells are needed (see Table 38).
- Stimulate the NLRP3 inflammasome as described in 9.3.2.1. (step 4–8). After addition of inflammasome stimulator ATP, incubate the plate at 37°C for 3–12 h dependent on the performed assay. The volume in each well should be 50 µl.
- 30 min before the incubation time ends, add 10 µl of Lysis buffer (containing Octoxinol (Triton X-100); Lysis buffer is part of the LDH-Cytox™ Assay Kit, BioLegend) to all high control wells (Well 1 of Table 37) and 10 µl of DC medium to all other wells.
- Centrifuge the plate with  $250 \times g$  for 2 min at RT. Transfer 50 µl of the supernatant into a new optically clear 96-well F-bottom plate. Add 50 µl of working solution (containing 2H-Tetrazolium, 5-(2,4-disulphophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-, inner salt, sodium salt (1:1); working solution is part of the LDH-Cytox™ Assay Kit, BioLegend) to each well. Incubate for 30 min protected from light.
- After the incubation time, add 25 µl of Stop solution (Stop solution is part of the LDH-Cytox™ Assay Kit, BioLegend) to each well. Measure the absorbance at 490 nm using an ELISA reader.

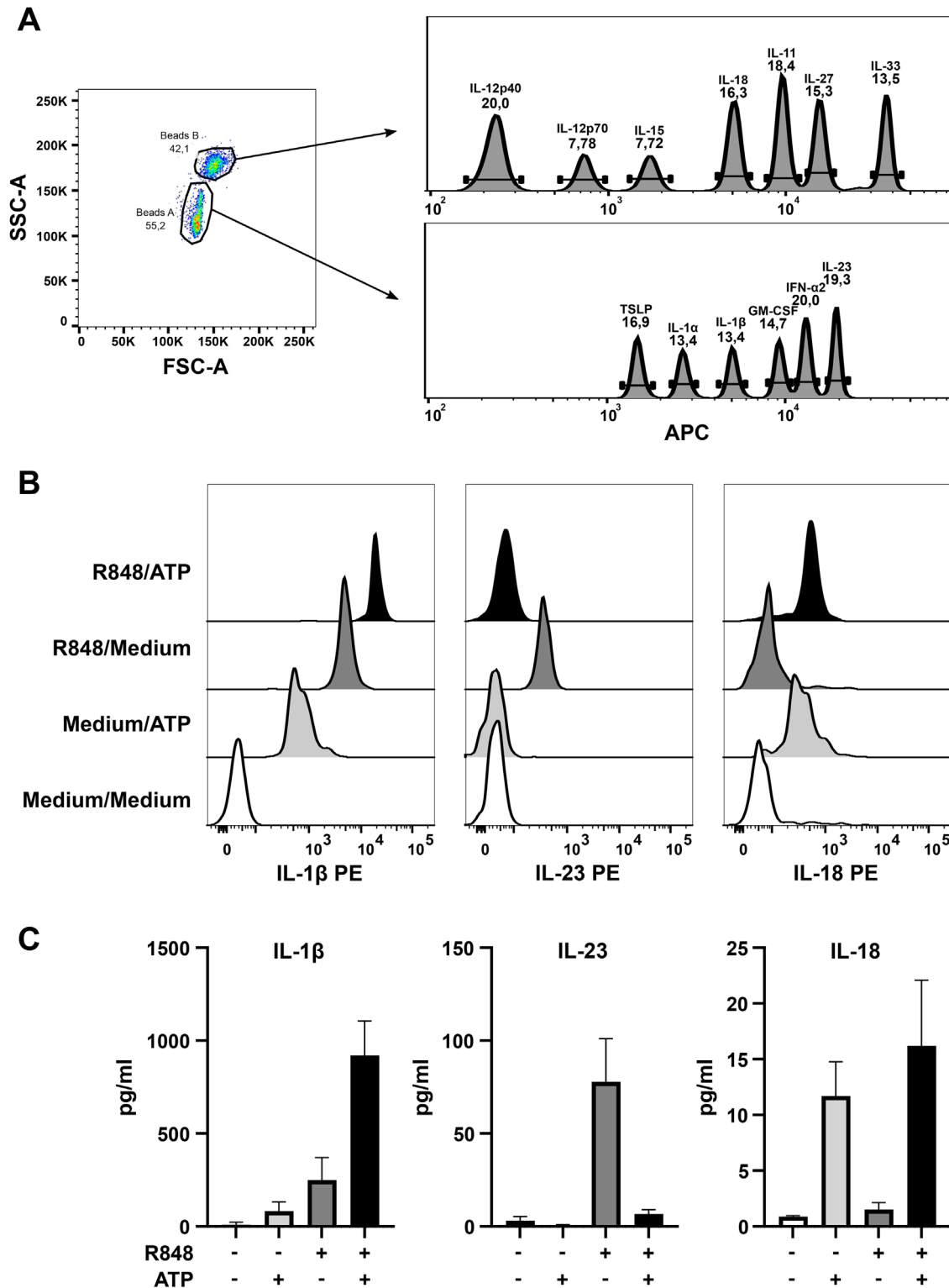
### 9.3.2.3 Measuring of active caspase-1.

- Isolate primary human cDC1, cDC2, pDC, and monocytes as described in section 3 “Cell sorting of primary human DC” in [156]. Resuspend the isolated DC in DC medium to a final concentration of  $1 \times 10^6$  cells/ml.
- Transfer  $1 \times 10^5$  DC or monocytes (100 µl of the prepared suspension in step 26) into a well of a sterile 96-well V-bottom plate. For each cell population to be analyzed, prepare four wells (see Table 36).
- Stimulate the cells with R848 as described in 9.3.2.1. (step 4). Incubate the plate at 37°C for 3 h. Shortly before the end of the incubation time, prepare a 30× FLICA solution by diluting the 150 x stock 1:5 in PBS. The diluted 30 x FLICA solution should be used within 30 min.
- After washing as described in 9.3.2.1. (step 5–7), add 174 µl of the prepared 5 mM ATP solution or DC medium as control as well as 6 µl of the 30× FLICA solution to each well. Resuspend the cells and incubate at 37°C for 3 h.
- Centrifuge the plate with  $520 \times g$  for 5 min at 4°C. Remove the supernatant and wash the cells with 150 µl of 1 x apoptosis wash buffer.
- Centrifuge the plate with  $520 \times g$  for 5 min at 4°C. Remove the supernatant, add 150 µl of FACS buffer containing DAPI (1:10,000) and acquire the cells using a BD LSRFortessa.

## 9.4 Data analysis

### 9.4.1 Measuring of secreted IL-1β

- Data of experiment 9.3.2.1 are analysed using the LEGENDplex™ Data Analysis Software Suite. In a first step, acquired beads are separated into Beads A and B dependent on size and granularity (FSC-A vs. SSC-A) (Fig. 22A).
- Then, individual beads are identified by APC signal as the different beads differ in the fluorescence intensity (Fig. 22A). Thereby, up to seven beads targeting different cytokines can be measured for both Beads A and B.
- On each gated bead, the fluorescence intensity in the PE channel is measured (Fig. 22B). By generating a standard curve



**Figure 22.** Measuring of secreted cytokines by ATP-treated human cDC2. Sorted human cDC2 were primed or not with 5  $\mu$ g/ml R848 for 3 h. Then, the inflammasome was activated or not with 5 mM ATP for 12 h. Subsequently, supernatants were harvested and stored at  $-80^{\circ}\text{C}$  until analysis using the LEGENDplex Human Cytokine 2 Panel. 4,000 beads per sample were acquired using an LSRFortessa. (A) Beads are separated into Beads A and B using FSC-A/SSC-A. Then, each bead population is analyzed for signals in the APC channel and beads are divided into six (Beads A) or seven (Beads B) individual bead populations binding a specific cytokine. (B) Exemplary histogram gates for beads binding IL-1 $\beta$ , IL-23, or IL-18. Overlay histograms (half-offset) show different experimental conditions. (C) Bar graphs show mean + SD of the concentration in the supernatant for IL-1 $\beta$ , IL-23, and IL-18 of four healthy blood donors.

**Table 39.** Summary of the results of the different experiments

Experimental condition	Read out of the assays		
	9.3.2.1 Cytokine secretion	9.3.2.2 LDH release	9.3.2.3 activation of caspase-1
Medium only	No cytokine secretion	No cell-death induced release of LDH	No activation of caspase-1
Priming with R848	Secretion of inflammatory cytokines with low levels of IL-1 $\beta$	No cell-death induced release of LDH	No activation of caspase-1
Priming with R848 and stimulation of NLRP3 with ATP	Secretion of inflammasome-dependent cytokines	High release of LDH due to pyroptosis	Cells contain active caspase-1 and undergo pyroptosis

using C0-C7, the concentration of the cytokines for each sample is calculated (Fig. 22C).

- In this experiment, cell-sorted cDC2 were stimulated as described above with R848 and the NLRP3 inflammasome activated with ATP. While the combination of R848 as priming signal and ATP as inflammasome ligand induce the secretion of the highest amounts of the inflammasome-dependent IL-1 $\beta$  and IL-18, the secretion of IL-23, which is induced by R848, is lost (Fig. 22B-C; Table 39).

#### 9.4.2 Measuring of inflammasome-induced pyroptosis

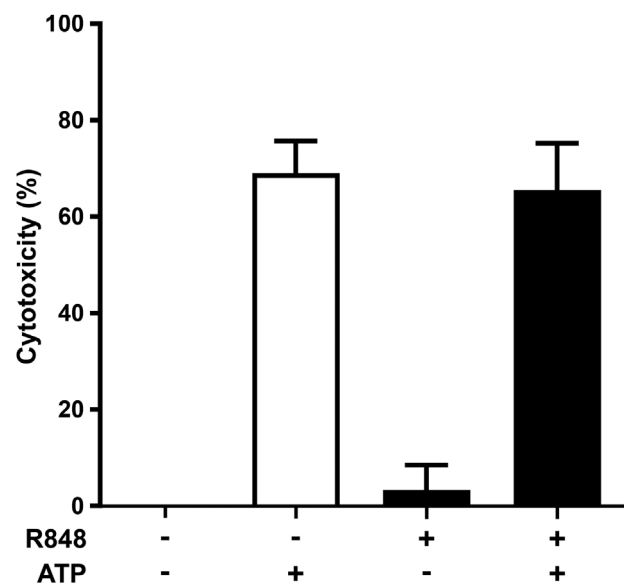
- Data of the experiment 9.3.2.2 are analyzed using Excel. During inflammatory cell death such as pyroptosis, lactate dehydrogenase (LDH), which is usually in the cytosol of a cell, is released into the supernatant due to rupture of the membrane. The LDH in the supernatant then catalyses dehydrogenation of lactate to pyruvate thereby reducing NAD<sup>+</sup> to NADH. NADH reduces water-soluble tetrazolium salt in the presence of an electron mediator to produce an orange formazan dye. The absorbance (OD) of the orange formazan dye is measured and is proportional to the released LDH. Thereby, cytotoxicity is measured.
- In order to determine how much cell death occurred, positive (high control) and negative (low control) controls have to be performed and are used for the calculation of induced cell death using this formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{OD (sample)} - \text{OD (low control)}}{\text{OD (high control)} - \text{OD (low control)}} \times 100$$

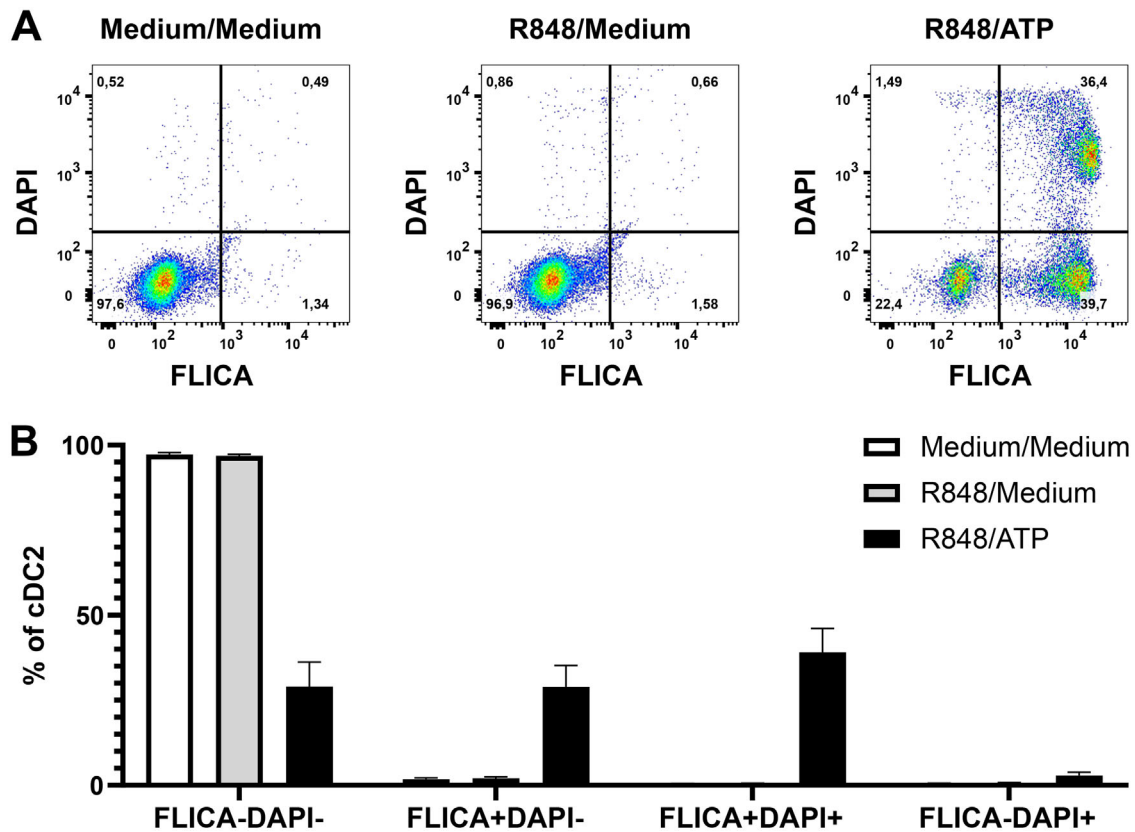
- In this experiment, cell-sorted cDC2 were stimulated as described above with R848 and the NLRP3 inflammasome ligand ATP. While medium or stimulation with R848 alone did not induce measurable cell death, the NLRP3 inflammasome ligand ATP induced pyroptosis in almost all cells irrespective of priming with R848 or not (Fig. 23; Table 39).

#### 9.4.3 Measuring of active caspase-1

- FLICA is cell-permeant and will efficiently diffuse in and out of all cells. If there is active caspase-1 inside the cell, it will covalently bind with FLICA and retain the green fluorescent signal within the cell. Unbound FLICA will diffuse out of the cell during the washing steps. Pyroptotic cells will retain a higher concentration of FLICA and fluoresce brighter than healthy cells.
- To further characterize the status of the cells, a live/dead staining was performed using DAPI. As DAPI is not cell-permeable, it is excluded from living cells, while it can bind to the DNA of cells with membrane pores or ruptures as it typically occurs during pyroptosis. Therefore, the acquired



**Figure 23.** Measuring of ATP-induced cell death using an LDH-cytotoxicity assay. Sorted human cDC2 were primed or not with 5  $\mu$ g/ml R848 for 3 h. Then, the inflammasome was activated or not with 5 nM ATP for 12 h. Subsequently, supernatants were harvested and analyzed using the LDH-cytox Assay Kit. Absorbance (OD) was measured using a VersaMax ELISA plate reader (Molecular Devices) and cytotoxicity determined using the formula described in 9.4.2. (step 6). Bar graphs show mean + SD of five healthy blood donors.



**Figure 24.** Detection of active caspase-1 in ATP-treated human cDC2. Sorted human cDC2 were primed or not with 5  $\mu$ g/ml R848 for 3 h. Then, the inflammasome was activated or not with 5 nM ATP for 3 h. Subsequently, cells were stained with DAPI (1:10,000) and samples were acquired using an LSRFortessa and data analyzed using FlowJo software. (A) Pseudocolor dot plots show fluorescence signals in the FLICA and DAPI channels for the different experimental conditions. Cells are divided into FLICA<sup>-</sup>DAPI<sup>-</sup>, FLICA<sup>+</sup>DAPI<sup>-</sup>, FLICA<sup>+</sup>DAPI<sup>+</sup>, and FLICA<sup>-</sup>DAPI<sup>+</sup> cells using a quadrant gate. (B) Bar graphs show mean + SD of the ratio of FLICA<sup>-</sup>DAPI<sup>-</sup>, FLICA<sup>+</sup>DAPI<sup>-</sup>, FLICA<sup>+</sup>DAPI<sup>+</sup>, and FLICA<sup>-</sup>DAPI<sup>+</sup> cells of four healthy blood donors as described in (A).

cells of assay 9.3.2.3. are analyzed using FlowJo software for the fluorescence intensity of FLICA and DAPI. After a morphology gate to exclude debris and selection of single cells using FSC-A vs. FSC-H, the remaining cells are analyzed for FLICA and DAPI. Using a quadrant gate, cells are divided into (Fig. 24A):

- FLICA<sup>-</sup>DAPI<sup>-</sup> cells, which are alive and did not activate caspase-1,
- FLICA<sup>+</sup>DAPI<sup>-</sup> cells, which activated caspase-1 but are not dead yet,
- FLICA<sup>+</sup>DAPI<sup>+</sup> cells, which activated caspase-1 and are already pyroptotic, and
- FLICA<sup>-</sup>DAPI<sup>+</sup> cells, which died but did not activate caspase-1 before.

- Sorted cDC2, which were cultured in medium or stimulated with R848 only, did neither show staining for FLICA nor DAPI (Fig. 24A-B; Table 39). Thus, the cells were alive and did not activate the inflammasome. However, after addition of ATP, the majority of the cells activated caspase-1 as measured by fluorescence in the FLICA channel. Further, half of the FLICA<sup>+</sup> cells were already pyroptotic (FLICA<sup>+</sup>DAPI<sup>+</sup>) as they stained brightly for DAPI in addition to FLICA (Fig. 24A-B;

Table 39). Also here, almost no cells died without activation of caspase-1 (FLICA<sup>-</sup>DAPI<sup>+</sup>).

## 9.5 Pitfalls

### Problem: Discrimination of bead populations is not possible (9.4.1)

#### Potential solutions:

To discriminate 13 beads populations based on FSC-A, SSC-A and APC signal, appropriate setup of the flow cytometer is very important. The setup for different flow cytometers, dependent on the laser equipment, is explained in the manufacturer instructions and should be performed as described. FSC-A and SSC-A have to be adjusted so that two clearly separated bead populations (see Fig. 22A) are visible. Using the included beads for setup of the flow cytometer, the APC signal should be adjusted in a way that the beads with the lowest fluorescence have a signal of  $1 \times 10^2$  and the beads with the strongest fluorescence should not exceed a signal of  $5 \times 10^4$  to ensure proper gating using the software. In case of problems with the separation of the beads, unused capture beads (see step 13 of section 9.3.2.1.2) should be acquired. If the



separation of all 13 bead populations is not possible, adjust either FSC-A/SSC-A to better segregate beads A from beads B (Fig. 22A, left panel) or adjust APC to improve the space between the single beads (Fig. 22A, right panel).

**Problem: The LEGENDplex software does not calculate the standard curve (9.4.1)**

**Potential solutions:**

Different issues can lead to problems with the calculation of the standard curve, such as the reconstitution of the top standard (C7), the serial dilution (C6-C1) as well as the setup of the flow cytometer:

**Reconstitution of the top standard (C7):** Make sure that the lyophilised cytokines are properly reconstituted. Pre-heat the lyophilised cytokines and the Assay buffer to RT. Add the Assay buffer to the lyophilised cytokines without vortexing and do not directly mix by pipetting up and down. Let it stand for at least 10 min before gently mixing using a pipette.

**Serial dilution:** For the standard curve, the top standard (C7) has to be diluted serially 1:4 by mixing 75  $\mu$ l of Assay buffer with 25  $\mu$ l of standard. After addition of 25  $\mu$ l of the top standard (C7) to the 75  $\mu$ l of Assay buffer (C6), mix the liquid in tube C6 properly by pipetting up and down several times with an appropriate pipette. As the tube contains 100  $\mu$ l set a pipette to at least 75  $\mu$ l to entirely mix it before removing 25  $\mu$ l for the next dilution (C5). Proceed analogously until C1. Be aware that C0 is a blank. Do not add 25  $\mu$ l of C1 to C0.

**Setup of the flow cytometer:** During setup of the flow cytometer for the acquisition of the samples, the PE channel, which is used for the calculation of concentration of the cytokines, should be adjusted to a mean value of  $65 \pm 5$  for the gated bead population. Make sure that the setup beads as well as the unstained capture beads of the kit (see step 13 of section 9.3.2.1.2) are in the range of  $65 \pm 5$  or adjust the voltage for the PE channel accordingly. After acquisition of the samples for the standard curve, do not directly throw away the tubes but keep them in case adjustments to the PE channel have to be performed. If the top standard during acquisition gives too strong signals in the PE channel (e.g., reaches the end of the X-axis), the software will not calculate the standard curve for these beads. Then, reduce the voltage for the PE channel even if it leads to lower mean values than 65 for the unstained beads. Re-acquire few beads of C7 to assure that they do not touch the end of the axis anymore. Repeat the acquisition of all standard samples (C0-C7). Before the acquisition of all samples, export the fcs files of the standard curve in order to test, whether calculation of the standard curve works now.

**Problem: The measured absorbance values are too low or too high (9.4.2)**

**Potential solutions:**

If the absorbance values in the high control are also too low or high, the number of cells used for the assay were inappropriate. The absorbance is proportional to the released LDH. As dependent on the cell size and the type of cell, the cytosolic amount of LDH might vary, the cell number has to be adjusted to the used cell

type. The protocol in this section works well for primary human DC subpopulations but has to be adapted if monocyte-derived DC are used. Here, the cell number in each well should be reduced to avoid too high values.

**Problem: ATP- or other inflammasome-stimulated samples do not contain any events (9.4.3)**

**Potential solutions:**

If only the samples, which were stimulated with an inflammasome ligand, did not contain any events, then pyroptosis might have occurred too fast. Repeat the experiments with shorter incubation times for priming (e.g., only 2 h R848; step 28 of section 9.3.2.3) and inflammasome stimulation (e.g., only 1h ATP; step 29 of section 9.3.2.3).

**Problem: No difference between inflammasome-stimulated and control-treated samples**

**Potential solutions:**

In case both control-treated as well as inflammasome-stimulated DC show high cell death in the culture, culture conditions might not be ideal for the cells. As LDH is released into the cell culture medium by different ways of cell death (e.g. necrosis and secondary apoptosis), cell culture conditions have to be optimized to avoid any cell death due to the culture of the cells in plates. The used medium is optimized for human primary DC isolated from blood and might have to be adapted if other cells are used. If working with monocyte-derived DC, the medium used for differentiation should be used for the assay. Further, only plates should be used that allow culture for at least 12h without induction of significant amounts of cell death. E.g., in case untreated plastic dishes induce activation of the DC, this could lead to cell death with higher background values. Further, if cells are activated due to the culture condition, this will impair differences between primed and not primed samples as the activation might induce expression of inflammasome genes.

## 9.6 Top tricks

This section describes the analysis of inflammasome responses in human DC after stimulation with ATP. However, as a vast array of inflammasome-activating ligands exist and also certain viruses as well as bacteria activate different subtypes of the inflammasome [142, 157–160], the protocol can be easily applied to other stimuli (see for example [153]). In order to proof that inflammasomes are activated in response to distinct ligands, inhibition of caspase-1 can be performed using ac-yvad-cmk (#inh-yvad, InvivoGen) [153]. Inhibition of caspase-1 should completely abolish the secretion of IL-1 $\beta$ . In case markedly amounts of IL-1 $\beta$  are still detectable, non-canonical inflammasomes using other caspases, such as caspase-4, -5 or -8, might be activated [161–167]. In addition, specific inhibitors for NLRP3, such as MCC950 (#inh-mcc, InvivoGen), exist, which can be used to test which inflammasome subtype is activated in response to newly identified or not well-characterized inflammasome ligands [153].

In addition to human primary DC isolated from blood, the described assays can be performed with DC isolated from human lymphoid tissues such as spleen. In case the DC are prematured due to the isolation protocol, inflammasome activation with subsequent pyroptosis and cytokine secretion might be observed without an additional priming step. However, using our adjusted protocols for isolation of lymphoid tissue DC (see section 1 **Generation of single cell suspensions from lymphohematopoietic tissues** in [156]), inflammasome activation is not observed without TLR-induced priming and subsequent inflammasome activation.

## 9.7 Summary table

This section describes assays to measure inflammasome activation in primary human dendritic cells using different readouts, such as release of inflammasome-dependent cytokines (9.3.2.1), LDH (9.3.2.2) or measuring active caspase-1 (9.3.2.3). The results for human cDC2 stimulated with the NLRP3 ligand ATP after priming with R848 are summarized in Table 39.

## 10 In vitro antigen-presentation assays with murine DC and CD4<sup>+</sup> T cells

### 10.1 Introduction

#### 10.1.1 DC as antigen presenting cells

Dendritic cells (DC) are professional antigen-presenting cells (APC) and are well equipped with MHC-class II molecules (MHCII) and T cell costimulatory molecules, such as CD80 and CD86. In order to present antigens and to stimulate cognate CD4<sup>+</sup> T cells, the first step is antigen uptake [168]. DC possess three major possibilities to take up antigens: 1. by phagocytosis (uptake of solid antigens), 2. by pinocytosis (“drinking”; uptake of antigens soluble in liquids), and 3. by absorptive endocytosis. That is, DC express defined surface receptors (see review by Stoizner et al., this issue) that are capable to bind defined ligands and to mediate endocytosis and further intracellular routing to MHCII<sup>+</sup> compartments via coated vesicles. Following endocytosis, ingested antigens have to access defined late endosomal/lysosomal compartments where loading of MHC class II molecules occurs [169]. These endocytic compartments are also referred to as MHCII compartments (MIICs) and are constitutively synthesized by DC. In MIICs antigens are chopped into a variety of peptides of 13–25 amino acids in length and are bound to the binding-groove of newly synthesised MHCII in exchange for the CLIP peptides that serve as final placeholder for antigenic peptides in the fabrication process of MHCII. The now fully loaded MHCII:peptide complexes are transported back to the surface and are now assessable for binding to cognate T cell receptors [170, 171]. Nevertheless, that alone is not sufficient to initiate T cell proliferation, as T cell costimulatory signals (signal 2) have to

be provided to the T cells once the antigen is presented. Therefore, among all subtypes of APC DC are most efficient in expressing a multiplicity of co-activation molecules such as CD80, CD86, CD83, CD40, ICOS ligand [172]. Thus, with the right mixture of MHC-II peptide complexes and T cell costimulatory molecules, DC act as pivotal stimulators of an immune response.

However, this is a very sketchy plot of how antigen presentation by DC occurs, as the antigen presentation process and the resulting T cell proliferation can be fine-tuned by several means. For instance, in activated DC antigen uptake (regulated by differential receptor expression and/or re-direction of intracellular receptor routing) and loading of MHCII molecules (increased synthesis and enhanced loading/transport to the surface) is effectively upregulated, leading to 10 to 1000-fold more MHCII:peptide complexes reaching the cell surface as compared to non-activated DC. To make regulation of the immune response even more complex, DC are also capable of expression inhibitory molecules such as PD-1 LAG3, BTLA4, which will counteract the initial T cell activation guided by MHCII:peptide complexes. And of course, the secretion of pro- or anti-inflammatory cytokines by differentially activated DC adds another facet to the regulatory mechanisms of the DC-T cell interactions during antigen presentation. Therefore, to test the antigen presentation capacity of DC in vitro is a valid parameter to assess their physiologic function that cannot be substituted by measuring surface molecule expression or cytokine secretion. In the following, we provide a protocol using transgenic OTII mice to set up a non-radioactive in vitro assay for DC assessment.

#### 10.1.2 The choice of responder T cells

Bulk T cells are used, i.e., we will not distinguish between naive and memory T cells. Of course, the animals are naive, however that does not mean that all T cells are truly naive as assessed by being CD44 negative. Indeed, memory-phenotype T cells respond to antigen in ways different from naive-phenotype T cells [173]. Memory T cells proliferate in response to lower concentrations of antigen, do not require a long antigenic stimulation as compared to naive T cells, display effector functions (i.e., IL-2 secretion) sooner after activation and they are less dependent on costimulatory signals than naive T cells. Thus, in vivo activation of naive T cells is strictly dependent on dendritic cells (DC), while memory T cells also respond to antigen presented by other APC. But by using DC as stimulators in vitro, there are only marginal differences in the proliferative response of naive and memory-type T cells, and the assay is suitable to generate a quantitative overview of the T cell stimulatory capacity of the DC subtypes used in the respective assays. Nevertheless, the researcher has to determine whether truly naive T cells are necessary for the individual assay.

#### 10.1.3 The choice of viable or fixed DC

In the basic assay viable DC are used as stimulators, but as DC as well as the responder T cells are viable, mutual interactions

**Table 40.** Summary of standard chemicals for proliferation assays

Chemicals and Reagents	Company	Ordering number
Albumin Fraktion V, proteasefrei	Carl Roth, Karlsruhe, Germany	
CD4 <sup>+</sup> T cells Isolation Kit mouse	Miltenyi Biotec, Bergisch Gladbach, Germany	130-104-454
eBioscience™ Cell Proliferation Dye eFluor™ 450	Thermo Fisher Scientific, Dreieich, Germany	65-0842-85
Fetal calf serum (FCS)	Gibco, Darmstadt, Germany	10500-064
HEPES, ≥99.5%	Carl Roth, Karlsruhe, Germany	
L-Glutamine 200 mM	Sigma, Taufkirchen, Germany	
Minimum Essential Medium (MEM)	Gibco, Darmstadt, Germany	
OVA peptid (323-339)	Genaxxon, Ulm, Germany	P3287.9505
OVA protein	Merk, Darmstadt, Germany	
PBS Dulbecco w/o Ca <sup>2+</sup> w/o Mg <sup>2+</sup>	Biochrom, Berlin, Germany	
Penicillin/ Streptomycin (100×)	Sigma, Taufkirchen, Germany	
RPMI 1640	Bio&Sell, Feucht, Germany	
Trypan Blue Stain, 0.4%	Gibco, Darmstadt, Germany	
Zombie Aqua™ Fixable Viability Kit	Biolegend, Koblenz, Germany	423101
Anti-CD3, Biotin	Biolegend, Koblenz, Germany	100304
Anti CD28, Biotin	Biolegend, Koblenz, Germany	102116
Anti CD4, PE	Biolegend, Koblenz, Germany	100512

**Table 41.** Additional cell proliferation dyes

Cell tracker	Company	Ordering number	Reference
CellTrace™ CFSE Cell Proliferation Kit	Thermo Fisher Scientific, Dreieich, Germany	C34554	[175]
CellTrace™ Violet cell proliferation kit	Thermo Fisher Scientific, Dreieich, Germany	C34557	[176]
Tag-It Violet Proliferation Cell Tracking Dye	Biolegend, Koblenz, Germany	425101	[177]
Oregon Green 488, SE	Thermo Fisher Scientific, Dreieich, Germany	C34555	[178]
eBioscience™ Cell Proliferation Dye eFluor™ 670	Thermo Fisher Scientific, Dreieich, Germany	65-0840-90	[179]

can take place and the phenotype of DC may change during culture. For instance, T cells can augment the expression of T-cell co-stimulatory molecules by DC, which in turn enables them to stimulate proliferation of T cells rather antigen unspecific [174]. Moreover, different cytokine profiles of T cells (as a mixture of naïve and memory like phenotype T cells may be used in the assays), may drive differentiation of DC in culture to a more or less stimulatory phenotype. But as these effects may occur in parallel in all different test samples, the results obtained for T cell proliferation are valid. However, under certain circumstances it may be important to freeze the status of the stimulatory DC to avoid any changes and interactions with T cells in vitro. To this end DC can be fixed with paraformaldehyde (PFA) prior to setting up the stimulatory assays. For that reason, a subsection of how to fix DC is included into the protocol.

## 10.2 Materials

### 10.2.1 Chemicals and reagents

Table 40 summarizes all chemicals used for the assays. They were purchased as cell culture grade, if available. If not otherwise stated the highest purity was obtained.

In Table 41 all cell proliferation dyes currently sold are listed. All were used according to the manufacturer's protocols. So please note to adjust cell density and washing steps.

### 10.2.2 Consumables

All consumables are listed in Table 42. Some of the consumables used for these experiments are sold from different vendors. It is not mandatory to buy each part from the exact same vendor. For example, it is not necessary to buy all plastic ware from Greiner.

### 10.2.3 Devices

The devices listed in Table 43 are produced from different companies. It is not necessary to buy this equipment from the exact same vendor that is listed here. These are just examples from our laboratory.

### 10.2.4 Buffers and solutions

The buffers and solutions listed in Table 44 can be purchased as ready to use solutions. However, for the experiments shown in

**Table 42.** Standard equipment for proliferation assays

Consumables	Company
Falcon 5ml Polystyrene Round-Bottom Tube (12×75mm); FACS tubes Costar 96 well cell culture round bottom plates well Cell strainers (40 μm, 70 μm) Magnetic LS Columns Conical tubes, 15 ml /50 ml Sterile filter (0.2 μm) Pipette Tips (10 μl, 200 μl, 1000 μl) Costar Pipettes (1 ml, 5 ml, 10 ml, 25 ml)	Corning Lifescience, Amsterdam, Netherlands Corning LifeScience, Amsterdam, derlandsNetherlandsork, USA Corning Lifescience, Amsterdam, Netherlands Greiner, Frickenhausen, Germany Miltenyi Biotec, Bergisch Gladbach, Germany Greiner, Frickenhausen, Germany Sartorius, Göttingen, Germany Greiner, Frickenhausen, Germany Corning Lifescience, Amsterdam, Netherlands Corning, New York, USA

this section, the buffers were prepared from solid chemicals. If not otherwise stated all solutions were sterile filtered and kept at 4°C in the fridge for up to 4 weeks.

### 10.3 Step-by-step sample preparation

#### 10.3.1 Preparation of single-cell suspensions from lymph nodes and spleen

Note: You have you better yields when isolating spleen cells and LN cells separately.

##### 10.3.1.1 Lymph nodes.

1. Sacrifice an OTII (B6.Cg-Tg(TcraTcrb)425Cbn/J) mouse and collect lymph nodes (cervical, inguinal, axillary) into 10 ml MEM medium (media and buffers are listed in Table 40 and preparation is depicted in Table 44) in a 50 ml conical tube (consumables are listed in Table 42).
2. Grind through a 70 μm cell strainer.
3. Fill up with 20 ml MEM medium.

**Table 43.** Technical equipment for proliferation assays

Device	Company
Benchtop pH meters pH 50 VioLab	Carl Roth, Karlsruhe, Germany
Centrifuge Multifuge 3 S-R	Thermo Fisher Scientific, Dreieich, Germany
CO <sub>2</sub> Incubator MCO-20AIC Spectrophotometer/ Fluorometer	Sanyo Electric, Tokyo, Japan Germany
Gallios flow cytometer	Beckman Coulter, Krefeld, Germany
MACS® MultiStand	Miltenyi Biotec, Bergisch Gladbach, Germany
Vortex Reax top	Heidolph Instruments, Schwabach, Germany

4. Centrifuge at 365×g for 7 min at 4°C (technical equipment, ie. centrifuges etc are listed in Table 43).
5. Discard the supernatant, resuspend cell pellets with 1ml MACS buffer.
6. Fill up with 20 ml MACS buffer.
7. Centrifuge at 365 × g for 7 min at 4°C.
8. Discard the supernatant, resuspend cell pellets with 10 ml MACS buffer.
9. Filter cells via a 70 μm cell strainer.
10. Count cells.
11. Fill up with 40 ml MACS buffer.
12. Centrifuge at 365 × g for 7 min at 4°C.
13. Discard supernatant, resuspend in MACS buffer to 1×10<sup>7</sup> cells per 40 μl MACS buffer.

##### 10.3.1.2 Spleens.

1. Put spleens into 10 ml MEM medium in 50ml conical tube.
2. Grind through a 70 μm cell strainer.
3. Fill up with 40 ml MEM medium.
4. Centrifuge at 365 × g for 7 min at 4°C.
5. Discard the supernatant, resuspend cell pellets with 3 ml ACK Lysis buffer per spleen for 3 min at RT.
6. Fill up with 40 ml MEM medium.
7. Centrifuge at 365g for 7min at 4°C.
8. Discard the supernatant, resuspend cell pellets with 1 ml MACS buffer.
9. Fill up with 40 ml MACS buffer.
10. Centrifuge at 365 × g for 7min at 4°C.
11. Discard the supernatant, resuspend cell pellets with 10 ml MACS buffer per spleen.
12. Filter cells via a 70 μm cell strainer.
13. Count cell numbers.
14. Fill up with 40 ml MACS buffer.
15. Centrifuge at 365 × g for 7 min at 4°C.
16. Discard the supernatant, cell pellets are single-cell suspensions.



**Table 44.** Preparation of buffers

Buffer	Amount/ Concentration	Ingredient
ACK lysis buffer	1.658g	NH <sub>4</sub> Cl
	0.2g 0.0076g add to 0.20L (pH7.2)	KHCO <sub>3</sub> EDTA ddH <sub>2</sub> O
Complete medium	1×	RPMI 1640
	1%	L-Glutamine 200mM
	1%	Penicillin/ Streptomycin (100×)
	10%	FCS
EDTA (100mM)	1%	HEPES (1M)
	3.72g add to 0.10L (pH7.2)	EDTA ddH <sub>2</sub> O
FACS buffer	1×	PBS
	3%	FCS
HEPES (1M)	47.66g add to 0.20L (pH7.2)	HEPES ddH <sub>2</sub> O
	MACS buffer	1×
0.5%		BSA
2mM		EDTA
MEM medium		MEM
MEM medium	47.65g	NaHCO <sub>3</sub>
	9.25g	HEPES
	23.85g	Penicillin/ Streptomycin (100×)
	1%	2-Mercaptoethanol
PBS (1× 5L)	17.5μL add to 5L (pH7.2)	ddH <sub>2</sub> O
	47.75g	PBS Dulbecco w/o Ca <sup>2+</sup> w/o Mg <sup>2+</sup>
	add to 5.00L (pH7.2)	ddH <sub>2</sub> O
Trypsin lysis buffer	1×	PBS
	0.5% 5mM	Trypsin EDTA

### 10.3.2 Isolation of CD4<sup>+</sup> T cells from single-cell suspensions of spleens and lymph nodes (CD4<sup>+</sup> T cells Isolation Kit, Miltenyi Biotec<sup>1</sup>)

1. Add 10 μl biotin-antibody cocktail per 40 μl of 10<sup>7</sup> cells. Use more beads when you have more cells/volume but keep the ratio of cells, buffer volume, and antibody cocktail.
2. Mix well and incubate on the shaker for 10min at 4°C.
3. Resuspend cells with 30 μl MACS buffer per 10<sup>7</sup> cells (or scale up accordingly).

4. Add 20μl anti-biotin microbeads per 10<sup>7</sup> cells (or scale up accordingly).
5. Mix well and incubate on the shaker for 15 min at 4°C.
6. Fill up with 20 ml MACS buffer.
7. Centrifuge at 300 × g for 10min at 4°C.
8. Discard the supernatant, resuspend cell pellets in 5 ml MACS buffer.
9. Place LS columns in the magnetic field of a suitable MACS Separator.
10. Prepare column by rinsing with 3 ml MACS buffer.
11. Apply cell suspension onto the column.
12. Collect flow-through containing unlabeled cells, representing the enriched CD4<sup>+</sup> T cells.
13. Wash column with 3ml MACS buffer.
14. Collect unlabeled cells that pass through, representing the enriched CD4<sup>+</sup> T cells.

### 10.3.3 Labeling of Cell Proliferation Dye eFluor<sup>TM</sup> 450

Many protocols use CFSE labeling. That is still possible, however when using it side-by-side eFluor<sup>TM</sup> 450 we found it less toxic for the cells and a more intensive stain (8 rounds of proliferation are clearly visible). Please note the materials section. Different dyes (Table 41) are available, depending on your color-needs.

1. Wash cells two times with PBS to remove any serum. (Optional keep 1×10<sup>4</sup> cells in 300 μl FACS buffer in Facs tubes on ice as negative control).
2. Resuspend cells at 2× the desired final concentration in PBS (pre-warmed to room temperature). For example, if the final concentration of cells desired is 10×10<sup>6</sup>/ml, then resuspend cells at 20×10<sup>6</sup>/ml.
3. Prepare a 20 μM solution of cell proliferation dye eFluor 450 in PBS (pre-warmed to room temperature).
4. While vortexing cells, add an equal volume of the 20 μM dye solution prepared in step 4.
5. Incubate for 10 min at 37°C in the dark.
6. Stop labeling by adding 4–5 volumes of cold complete media (containing 10% FCS) and incubate on ice for 5 min.
7. Wash cells 3 times with complete media. Count and check viability by trypan blue exclusion (viability >85%). (Optional: Prepare approx. 1×10<sup>4</sup> cells/300 μl FACS buffer and test for successful labeling. Negative cells (step 1) serve as controls).

### 10.3.4 Setting up T Cell proliferation assays

#### 10.3.4.1 Ova-pulsed DC.

1. Stimulatory DC can either be in vitro prepared BMDC (see section 7.3.1) or can directly be isolated from tissues (see section 8.3 and 8.4). DC have to be loaded with Ova peptide 323–339 or with Ovalbumin protein. This can be used to elucidate the capability of DC to actively process antigen. When

using Ovalbumin protein, DC need to uptake and to process it intracellularly, whereas Ova peptide can be presented to T cell without processing. Thus, a rough distinction between mature (non-processing DC) and still premature (Ova protein processing) DC can be made. Loading is achieved by adding Ova peptide (concentration of 100 ng/ml) or Ova protein 100 µg/ml to DC cultures for 6 h–12 h before harvesting. Of note, leave some aliquots of DC without Ova pulse. This will give you the background proliferation of the T cells without cognate antigen presentation.

2. Harvest DC by careful pipetting (mature DC are sensitive to shear forces) and wash 3 times with complete medium. Count DC and suspend to a final volume of  $4 \times 10^5$  cells/ml in complete medium.
- 2a OPTIONAL if fixed DC will be used in the assays: To fix DC 4% PFA is used. Don't use PFA solutions provided by several companies, they are not pure enough and the pH is not correct. Prepare 4% PFA solution as follows:
  1. Place 450 ml of dH<sub>2</sub>O in a glass beaker. Heat to 60°C using a hot plate with stirring.
  2. While stirring, add 20 g of paraformaldehyde powder. Cover and maintain at 60°C.
  3. Add 5 drops of 2N NaOH (1 drop per 100 ml). The solution should clear within a couple of minutes (There will be some fine particles that will not go away). Do not heat solution above 70°C. PFA will break down at temperatures above 70°C.
  4. Remove from heat and add 50 ml of 10× PBS. Adjust pH to 7.2; you may have to add some HCl. Adjust to final volume of 500 ml. Use bottle top filter and place on ice. Cover with foil to protect from light. Aliquots may be frozen at –20°C and thawed as needed.
  5. Resuspend DC in PBS to  $5 \times 10^6$  cells per ml PBS and add an equal volume of 4% PFA to the tube. Mix (not vortex) them and incubate at room temperature for 20 minutes with intermittent shaking.
  6. Wash DC with complete medium 3 times and resuspend to concentrations (i.e. of  $4 \times 10^5$  cells/ml) as needed for the assay.
3. Prefill as many rows as you need of a 96-well round bottom plate with complete medium. Add to the first three wells 100 µl of the DC suspension (if possible, use multi-channel pipette). Make serial 1:2 dilutions by transferring 100 µl of the first 3 wells to the next. Mix the wells by pipetting 2-times up and down and transfer again. Discard 100 µl from the last 3 wells. That way you will have triplicates of  $2 \times 10^4$ ,  $1 \times 10^4$ ,  $5 \times 10^3$  and  $2.5 \times 10^3$  DC per row.

#### 10.3.4.2 T cell activation via anti-CD3 and anti-CD28 antibodies (positive control).

1. Prepare 96-well round bottom plates by labeling at least 3 wells with 'activated T cells' and 'non-activated T cells'
2. Coat the "activated T cell wells" with the anti-CD3 antibody by diluting the anti-CD3 antibody to 10 µg/mL in sterile PBS.

Add 100 µl/well of diluted antibody to the wells. Just add PBS to 'non-activated T cells'. Incubate plate at 5% CO<sub>2</sub> at 37°C for 2 hours.

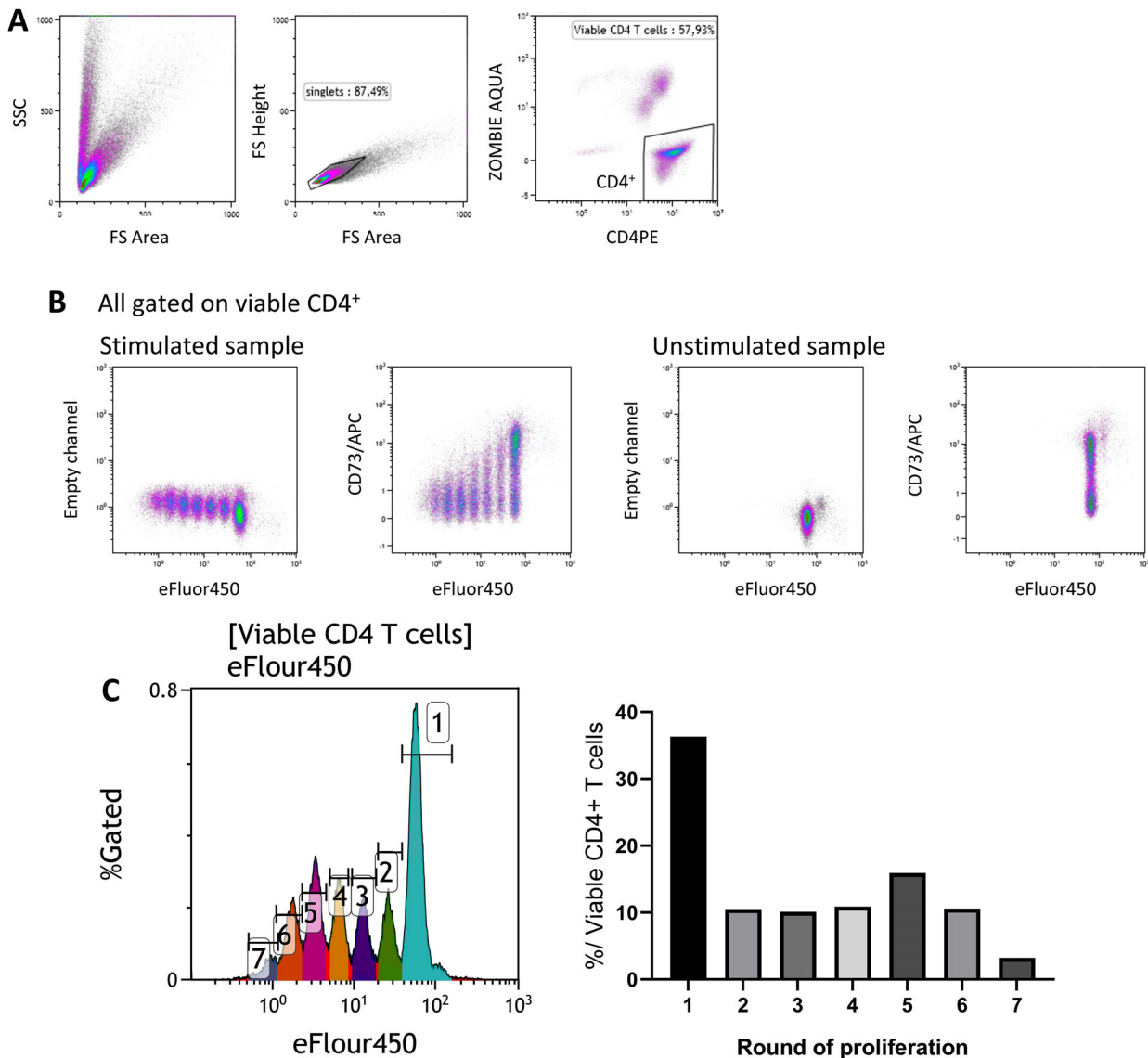
3. Retrieve 96-well plate from incubator after 2 hours. Aspirate the anti-CD3 and PBS solution and discard. Wash plate with 200 µl sterile cold PBS for each 'activated T cells' well. Finally, add 100 µl of complete medium to wells.

#### 10.3.4.3 Adding labeled T cells.

1. Dilute labeled T-cell suspension (see section 10.3.3) to a minimum of  $1 \times 10^6$ /ml.  $1 \times 10^6$  is rather the minimum. If available, we recommend to scale the number up to 1.2 –  $1.3 \times 10^6$  cell/ml. The proliferation is stronger.
2. Add 100 µl/well of the cell suspension to the DC plate. Prepare one triplicate of "T cells" only and add 100 µl complete medium. Each well should now be filled with 200 µl medium.
3. Add 100 µl/well of the cell suspension to the plate labeled 'activated T cells' and 'non-activated T cells'. Into the 'activated T cells' wells, add (maximally 10 µl) anti-CD28 to a final concentration of 1 µg/ml.
4. Incubate culture plate for 3–4 days in an incubator at 37°C, 5% CO<sub>2</sub>. Most T cells will require up to 3 days to divide. These cells will aggregate but will not be attached to the plate.

#### 10.3.5 Staining of CD4<sup>+</sup> T cells and analysis by flow cytometry

1. Harvest the T cell cultures by pipetting (200 µl) 2–3 wells to Polystyrene round-bottom tubes. Wash wells once with 200 µl of cold PBS and count cell numbers.
2. Wash cells with 3 ml PBS.
3. Dilute Zombie Aqua™ dye for live/dead discrimination (excitation by a violet laser) between 1:100–1:1000 in PBS. Resuspend  $1–10 \times 10^6$  cells in 100 µl of the diluted Zombie Aqua™ solution. To minimize background staining of live cells, titrate the amount of dye and/or number of cells per 100 µl for optimal performance. Different cell types can have a wide degree of variability in staining based on cell size and degree of cell death. In our cases, we dilute Zombie Aqua™ dye at 1:500 in PBS and use  $1 \times 10^6$  cells in 100 µl of a diluted Zombie Aqua™ solution. Incubate the cells at room temperature, in the dark, for 15–30 minutes.
4. Wash one time with 2 ml FACS buffer.
5. Stain  $1 \times 10^6$  cells with anti-CD4/PE (clone RM4-5) (0.2 µg) antibodies in 100 µl FACS buffer.  
OPTIONAL: (1) Additionally, you can stain the DC in the culture by using anti-MHC class II antibodies in a suitable color. E.g., add 0.1 µg anti MHC-II FITC or APC (clone M5/114) together with anti CD4. You can "gate out" DC in your analysis. However, it's not necessary as DC are „negative“ to „dim“ for CD4.



**Figure 25.** Example for analyzing proliferation of T cells by FACS. CD4<sup>+</sup> T cells pooled from spleen and lymph nodes were labeled with the proliferation dye eFluor 450 and cultured with anti-CD3 and anti-CD28 antibodies for 4 days. Controls as indicated were cultured without antibody stimulation. (A) Cells were stained with anti-Mouse CD4-PE and zombie aqua to discriminate live/dead cells and gated accordingly. (B) Viable (ZOMBIE AQUA<sup>-</sup>) CD4<sup>+</sup> cells were analyzed for eFluor F450 expression. Stimulated cells show a typical patterning indicating proliferation. In double labeling experiments with CD73 APC one can clearly visualize that activated, proliferating cells downregulate CD73 during cycling. In any case, unstimulated cells display no dilution of the dye. For further analysis individual eFluor 450<sup>+</sup> peaks can be gated in histograms (C) and the percentage of cells that had accomplished 1, 2, 3... rounds of proliferation can be depicted in a bar graph.

(2) Cells can easily be stained for other (activation) markers of interest, i.e., CD25, CD69, CD73. That way one can investigate whether the respective marker is up-, or downregulated during proliferation of the T cells. An example for downregulation of CD73 by activated/proliferated T cells is shown in Fig. 25.

6. Incubate for 30 min at 4°C
7. Fill tube with 3 ml FACS buffer.
8. Centrifuge at 365g for 7min at 4°C.
9. Discard the supernatant and resuspend cell pellets with 250  $\mu$ l FACS buffer.

10. Measure cells by Gallios flow cytometer (or alternative device).
11. Analyze data with Kaluza software (or alternative software).

#### 10.4 Data analysis

Cells were analyzed with a Gallios flow cytometer and data were visualized by Kaluza (BeckmanCoulter) software. In the Fig. 25 proliferation of CD4<sup>+</sup> T cells activated with anti-CD3 and anti-CD28 for 4 days is depicted. In case DC are used for stimulation, a population of CD4<sup>-</sup> cells will appear left of the CD4<sup>+</sup> gate.

## 11 Quantitative analysis of *in vitro* cross-presentation by mouse DC

### 11.1 Introduction

An adaptive immune response is initiated when dendritic cells (DC) recognize antigens in the peripheral tissue. Upon antigen recognition and subsequent internalization, DC migrate towards the draining lymph nodes, where they can activate antigen-specific T cells. For proper T cell activation, internalized antigens must be processed intracellularly and antigen-derived peptides are loaded onto major histocompatibility complex (MHC) molecules. Peptides loaded on MHC II molecules can be recognized by CD4<sup>+</sup> T helper cells, whereas CD8<sup>+</sup> cytotoxic T cells can be activated by peptides loaded on MHC I molecules. Presentation of extracellular antigens onto MHC I molecules is also termed cross-presentation [180].

Cross-presentation is mainly performed by cDC1 cells. It plays an important role in the initiation of an immune response against viruses that do not infect DC, against tumor cells of non-hematopoietic origin and in classical vaccination strategies aimed at the induction of a potent cytotoxic T cell response. The molecular mechanisms enabling cross-presentation, especially *in vivo*, are not completely solved yet and are still topic of intensive investigations [180–182].

Antigen cross-presentation can be monitored directly by flow cytometry, using specific antibodies that recognize the complex between a loaded peptide and MHC I molecule, or indirectly by measuring the proliferation or activation of antigen-specific CD8<sup>+</sup> T cells. Here, we will provide protocols for both direct and indirect analysis of cross-presentation of the model antigen ovalbumin (OVA) *in vitro*. We will describe how cross-presentation of OVA can be measured directly using the 25-D1.16 antibody. This antibody specifically recognizes the OVA epitope SIINFEKL (OVA<sub>257-264</sub>) when presented on the mouse MHC I in the context of H-2Kb [183]. Additionally, we describe how cross-presentation of OVA can be quantified after co-culture of OVA-loaded DC with OT-I cells, a specific CD8<sup>+</sup> T cell clone that recognizes the same OVA epitope on MHC I. After recognition of cross-presented OVA, OT-I T cells become activated, proliferate, and secrete IL-2. Both proliferation and the secretion of IL-2 are proportional to the amount of cross-presented OVA peptides and can be quantified using flow cytometry or ELISA. To measure proliferation, T cells are incubated with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) prior to their co-culture with antigen-treated DC. CFDA-SE is internalized by the T cells and cleaved by intracellular esterases into carboxyfluorescein succinimidyl ester (CFSE), which becomes membrane-impermeable and covalently links to cellular proteins. After each cell division, the fluorogenic CFSE is distributed among the daughter cells and fluorescence decreases accordingly. T cell activation can be easily monitored by determination of IL-2 in the supernatant of the cells using ELISA.

Naturally, these protocols can be easily adapted for other antigens / T cell clones.

### 11.2 Materials

#### 11.2.1 Reagents

A complete list of reagents is provided in Table 45.

#### 11.2.2 Equipment

Necessary equipment is listed in Table 46.

### 11.3 Step-by-step sample preparation

#### 11.3.1 Preparation of stocks and solutions

##### 11.3.1.1 2mM EDTA solution.

Prepare a 0.5 M EDTA stock by dissolving 146.12 g EDTA in 800 ml bi-distilled water. Adjust the pH to 8.0 with NaOH to enable dissolving. Fill up to 1000 ml with bi-distilled water and autoclave.

Add 2 ml of the 0.5 M stock to 500 ml sterile DPBS to generate 2 mM EDTA/PBS.

##### 11.3.1.2 mM $\beta$ -Mercaptoethanol solution.

Prepare a 5 mM  $\beta$ -mercaptoethanol solution by diluting 174.8  $\mu$ l  $\beta$ -mercaptoethanol in 500 ml DPBS and filter sterile.

##### 11.3.1.3 Cell culture media.

For BM-DC medium, add 10% FBS Premium, 50  $\mu$ M  $\beta$ -mercaptoethanol, Penicillin/Streptomycin (10 units/ml, 0.1 mg/ml) as well as 2.5% J558 supernatant (final concentration 25 ng/ml) to IMDM medium. J558 supernatant serves as GM-CSF source for differentiation of the cells.

For T cell medium, add 10% FBS Standard, 50  $\mu$ M  $\beta$ -mercaptoethanol and Penicillin/Streptomycin (10 units/ml, 0.1 mg/ml) to RPMI 1640 medium.

##### 11.3.1.4 ELISA buffers.

The ELISA wash buffer contains 0.05% Tween20 in DPBS.

To allow efficient immobilization of antibodies to positively charged ELISA plates, a coating buffer with basic pH is required. To prepare 1 l dissolve 8.401 g sodium bicarbonate in 950 ml bi-distilled water and check the pH value. If necessary, adjust to pH 8.2. Fill the volume with bi-distilled water to 1 l and store at 4°C.

For the ELISA blocking buffer add 1% BSA to DPBS.

0.2 M sulfuric acid can be prepared by diluting 0.55 ml 18 M sulfuric acid in 49.45 ml bi-distilled water.

Dilute 1 mg NaPox in 200  $\mu$ l bi-distilled water and fill up with 800  $\mu$ l DPBS to generate a stock solution of 1 mg/ml.

##### 11.3.1.5 FACS buffer.

Add 0.5% BSA to DPBS.



**Table 45.** Reagents, enzymes, chemicals and solutions

Reagent	Manufacturer	Ordering Number
<b>Antibodies</b>		
$\alpha$ -CD8-PerCP (53-6.7)	ThermoScientific	46-0081-80
$\alpha$ -CD11b-PE-Cy7 (M1/70)	Invitrogen	25-0112-82
$\alpha$ -mouse IL-2 purified (JES6-1A12)	eBioscience	14-7022-85
$\alpha$ -mouse IL-2 biotin-conjugated (JES6-5H4)	eBioscience	13-7021-85
$\alpha$ -SIINFEKL/H-2Kb clone 25-D1.16-PE	Biolegend	141604
$\alpha$ -CD11c-eFluor450	eBioscience	48-0114-82
<b>Chemicals &amp; Solutions</b>		
Mouse serum	PAN-Biotech	P30-0201M
PD10 desalting columns	Merck	GE17-0851-01
Dulbecco's Phosphate Buffered Saline (DPBS, w/o Ca <sup>2+</sup> and Mg <sup>2+</sup> )	PAN Biotech	P04-36500
RPMI 1640 medium	PAN Biotech	P04-22100
IMDM medium	PAN Biotech	P04-20150
Penicillin-Streptomycin	PAN Biotech	P06-07100
Fetal Bovine Serum (FBS) Standard	PAN Biotech	P30-3306
Fetal Bovine Serum (FBS) Premium	PAN Biotech	P30-1502
$\beta$ -Mercaptoethanol	Roth	4227.3
Ethylendiamintetraacetic acid (EDTA)	Roth	CN06.2
OVA	SERVA	11841.03
Trypan Blue	Sigma	T8154
CFSE	Invitrogen	65-0850-85
OT I peptide (SIINFEKL)	Anaspec	60193-1
Sodium bicarbonate	Roth	965.2
Tween20	Sigma Aldrich	P1379-500ml
DPBS powder (w/o Ca <sup>2+</sup> and Mg <sup>2+</sup> )	PAN Biotech	P04-36050P
Albumin Fraction V (BSA)	Roth	8076.4
TMB One	Kem-En-Tec	4395
Mouse IL-2 recombinant protein	eBioscience	148021-64
Sulfuric acid (H <sub>2</sub> SO <sub>4</sub> )	Honeywell Fluka	30743-1L-GL
CD8a (Ly-2) MicroBeads mouse kit	Miltenyi Biotec	130-117-044
NeutrAvidin Horseradish Peroxidase conjugate (NaPox)	ThermoScientific	A2664
70 $\mu$ m cell strainer	Greiner Bio-One	542070
40 $\mu$ m cell strainer	Greiner Bio-One	542040
5 mL syringe	Braun	4616057V
10 mL syringe	Braun	4616103V
Petri dish (10 cm)	Sarstedt	82.1135.500
96 well plate	Greiner Bio-One	655182
FACS Tubes	Sarstedt	55.1579
Cannula sterican G26 $\times$ 1", $\phi$ 0.25 $\times$ 40 mm	Braun	4657683

(Continued)

**Table 45.** (Continued)

Reagent	Manufacturer	Ordering Number
MS column	Miltenyi Biotec	130-042-201
ELISA Plate F Form	Greiner Bio-One	655061
Cell scraper	Sarstedt	83.3950

**Table 46.** Necessary equipment

Equipment	Company	Purpose
Scissors and tweezers	Hartenstein	Preparation of bones and tissue from mice
Microplate reader Infinite 200 PRO	Tecan	ELISA
BD LSR II Flow Cytometer	BD Biosciences	Flow cytometry

### 11.3.1.6 MACS buffer.

MACS buffer contains 0.5% BSA and 2 mM EDTA in DPBS (pH 7.2).

### 11.3.1.7 Purification of OVA.

Before its application in cross-presentation experiments, we clear OVA from small degradation products by gel filtration using PD-10 Sephadex-25 desalting columns. By this means, one can avoid background signals due to alternative presentation or direct loading of small peptide fragments.

1. Dissolve 300 mg OVA in 3 ml PBS in a 50 ml reaction tube by gently swinging the reaction tube.
2. Centrifuge dissolved OVA 5 min at 3200  $\times$  g
3. Wash a PD-10 Desalting Column 5 times with 4 ml DPBS. Add the supernatant onto the column and discard the flow-through.
4. Elute OVA from the column with 2.5 ml DPBS.
5. Determine OVA concentration at 280 nm with a UV-Spectrophotometer.

### 11.3.2 Generation of bone marrow-derived DC (BM-DC)

1. Sacrifice C57BL/6J mice by cervical dislocation, fix at the extremities and disinfect the ventral area of the chest, abdomen, and hind legs.
2. Starting from the lower half of the abdomen, cut open the skin towards the chest and fix on each side. Starting from the same point, cut open the skin across both hind legs, exposing the underlying tissues. After removing as much muscle and other connective tissue from the legs as possible, proceed by dislocating the femur from the pelvis and the tibia from the ankle, allowing excision of the leg bones without exposing the bone marrow to the unsterile environment. Submerge the leg bones

in DPBS, then transfer to a DPBS-filled petri dish in a sterile environment for removal of any excess soft tissue either by cutting action or friction using a paper tissue. SeverSeparate the femur from the tibia by overextension at the knee and cut open the resulting bone ends where necessary without sacrificing significant amounts of bone marrow.

3. Transfer the bones to a fresh DPBS-filled petri dish and fill a 10 ml syringe with DPBS. Using the syringe and a cannula (G26), flush out all visible bone marrow, making sure to do so from both ends of each bone to maximize yield. Resuspend the cells, pass them through a 40  $\mu\text{m}$  cell strainer, and centrifuge at  $300 \times g$  for 5 min. Take up the cell pellet in warm BM-DC medium and plate the cells onto three 10 cm Petri dishes (12 ml per dish). Incubate the cells at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .
4. Split the cells from each dish onto two fresh dishes on days 3 or 4 post-isolation. To do so, collect the supernatant and incubate the remaining adherent cells in 2mM EDTA solution at  $37^\circ\text{C}$  for 5 min. Wash the cells off the dish using a micropipette and combine with the supernatant. Centrifuge the resulting cell solution at  $300 \times g$  for 5 min, then aspirate the supernatant and take up the cell pellet in fresh BM-DC medium for plating.
5. The cells differentiate over the course of seven days, upon which they can be used for experimentation at a total yield of roughly  $1.2\text{--}1.8 \times 10^8$  BM-DC per mouse.

### 11.3.3 Quantification of OVA cross-presentation by flow cytometry using the 25-D1.16 antibody

For staining experiments, collect all BM-DC from one dish

1. Collect the supernatant of one petri dish in a 50 ml tube
2. Add 5 ml 2 mM EDTA solution to each plate, incubate for 5 min at  $37^\circ\text{C}$
3. Harvest cells from plate and add them to the supernatant
4. Centrifuge for 5 min at  $300 \times g$  and discard the supernatant
5. Resuspend the cells in BM-DC medium, count cells, and adjust the cells to  $10^6$  cells/ml
6. Plate 300  $\mu\text{l}$  (300.000 cells) per well in a 24-well plate
7. Incubate for at least 45 min at  $37^\circ\text{C}$
8. Add 100  $\mu\text{l}$  medium containing OVA (final concentration of 5 mg/ml) or peptide (final concentration of 400 nM), incubate for 6 h at  $37^\circ\text{C}$
9. Remove supernatant, add 500  $\mu\text{l}$  2mM EDTA solution per well
10. Harvest cells using a cell scraper
11. Collect cells in FACS tube, centrifuge for 5 min at  $300 \times g$
12. Wash cells with 2 ml FACS buffer, centrifuge for 5 min at  $300 \times g$ , discard the supernatant by decanting
13. Prepare antibody mastermix  
FACS Buffer containing Mouse serum (1:32 diluted), fluorochrome-conjugated 25-D1.16 antibody (1:30 diluted; final concentration 6  $\mu\text{g}/\text{ml}$ ),  $\alpha\text{CD11c}$  (1:500 diluted; final concentration 0.4  $\mu\text{g}/\text{ml}$ )

14. Add 50  $\mu\text{l}$  antibody master mix per tube
15. Incubate for 30 min at  $4^\circ\text{C}$
16. Wash cells with 2 ml FACS buffer, centrifuge for 5 min at  $300 \times g$
17. Collect cells in 300  $\mu\text{l}$  FACS buffer for analysis by flow cytometry

### 11.3.4 Quantification of cross-presentation after co-culture with antigen-specific T cells by ELISA

Stimulation of BM-DC with OVA or SIINFEKL

1. Harvest cells as in 3.3 using 2 mM EDTA
2. Count the cells and resuspend them to a concentration of  $2 \times 10^6/\text{ml}$  in BM-DC medium. Plate  $1 \times 10^5$  cells (50  $\mu\text{l}$ ) in a 96-well plate and incubate for at least 45 min at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .
3. Stimulate cells with different OVA concentrations (0, 10, 100, 200, 1000  $\mu\text{g}/\text{ml}$ ) or with the OT-I peptide (final concentration 100 ng/ml). To do this, add another 50  $\mu\text{l}$  medium with OVA or SIINFEKL in double concentration. Use freshly purified OVA as described in 11.3.1.7.
4. Incubate for 2 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

Preparation of OT-I cells

1. Sacrifice an OT-I mouse by cervical dislocation and isolate the spleen. The following steps are performed under sterile conditions.
2. Mash the spleen through a 70  $\mu\text{m}$  cell strainer in a petri dish filled with DPBS with the back of a 5 ml syringe.
3. Separate the cells by pipetting up and down and filter the suspension through a 40  $\mu\text{m}$  cell strainer to remove remaining clumps. Centrifuge the cell suspension at  $450 \times g$  for 10 min and resuspend the cells in 10 ml T cell medium. Count cells and centrifuge again at  $450 \times g$  for 10 min at  $4^\circ\text{C}$ .
4.  $\text{CD8}^+$  T cells were positively selected using the CD8a (Ly-2) MicroBeads mouse. Resuspend the cell pellet in 90  $\mu\text{l}$  MACS buffer per  $10^7$  cells and apply 10  $\mu\text{l}$  of CD8a (Ly-2) MicroBeads per  $10^7$  total cells. Mix and incubate the cells for 15 min at  $4^\circ\text{C}$ .
5. Wash the cells with 2 ml of MACS buffer and centrifuge cells at  $300 \times g$  for 10 min. Discard supernatant and resuspend the cell pellet in 500  $\mu\text{l}$  MACS buffer.
6. Place the MS column into an appropriate separator and rinse the MS column with 500  $\mu\text{l}$  MACS buffer. Transfer labeled cells onto the column and discard flow-through.
7. Wash the column 3 times with 500  $\mu\text{l}$  MACS buffer and discard flow-through
8. Remove the column from the separator and elute cells with 1 ml MACS buffer and a plunger. Collect flow-through and determine the cell number. Centrifuge at  $300 \times g$  for 10 min. Adjust the cells to a concentration of  $2 \times 10^6/\text{ml}$  in T cell medium.

### Co-culture with T cells

1. After incubation with OVA/SIINFEKL, remove the medium from the BM-DC and add  $2 \times 10^5$  purified T cells (100  $\mu$ l) for co-culture.
2. After 18 h, transfer the supernatant onto a new 96-well plate and store the plate at  $-80^\circ\text{C}$  until further analysis by ELISA.

### ELISA

1. Coat a high-binding 96-well plate (ELISA plate) overnight (ON) with 50  $\mu$ l/well of coating antibody (final concentration of 0.5  $\mu$ g/ml) in coating buffer. Store the plate ON at  $4^\circ\text{C}$ .
2. After ON incubation, wash the ELISA plate 3 times with 150  $\mu$ l wash buffer.
3. Add 100  $\mu$ l/well blocking buffer and incubate for 1 h at RT.
4. Wash the plate 3 times with wash buffer. Ensure that no liquid remains before continuing.
5. Prepare a 1:2 dilution series of mouse IL-2 recombinant protein in blocking buffer, ranging from 0.12 ng/ml to 125 ng/ml. Load 50  $\mu$ l sample and 50  $\mu$ l of IL-2 standard range as well as medium as a negative control (Blank).
6. Incubate the plate for 2 h at RT and wash three times with wash buffer.
7. Add 50  $\mu$ l/well biotinylated detection antibody (diluted 1:1000 in blocking buffer with a final concentration of 0.5  $\mu$ g/ml). Incubate for 1 h at RT.
8. Wash the plate thrice again in washing buffer.
9. Add 50  $\mu$ l/well Neutravidin (diluted in PBS with a final concentration 1  $\mu$ g/ml) and incubate for 30 min at room temperature.
10. Wash 3 times and add 50  $\mu$ l/well TMBOne substrate solution.
11. When the plate is sufficiently developed and a color change can be seen, stop the reaction with 50  $\mu$ l/well 0.2 M  $\text{H}_2\text{SO}_4$ .
12. Determine the optical density at 450 nm wavelength using a spectrophotometer.

### 11.3.5 Monitoring proliferation of activated T cells by flow cytometry

#### Stimulation of BM-DC with OVA or SIINFEKL

1. Harvest cells as in 3.3 using 2 mM EDTA
2. Count the cells and resuspend them to a concentration of  $1 \times 10^6$ /ml in BM-DC medium. Plate  $5 \times 10^4$  cells (50  $\mu$ l) in a 96-well plate and incubate for at least 45 min at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .
3. Stimulate cells with different OVA concentrations (0, 100, 1000  $\mu$ g/ml) or with the OT-I peptide (final concentration 100 ng/ml). To do this, add another 50  $\mu$ l medium with OVA or SIINFEKL in double concentration. Use freshly purified OVA as described in 11.3.1.7.
4. Incubate for 2 h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

### T cell isolation and CFSE staining

1. Sacrifice an OT-I mouse by cervical dislocation and isolate the spleen. The following steps are performed under sterile conditions.
2. Mash the spleen through a 70  $\mu$ m cell strainer in a petri dish filled with DPBS with the back of a 5 ml syringe.
3. Separate the cells by pipetting up and down and filter the suspension through a 40  $\mu$ m cell strainer to remove remaining clumps. Centrifuge the cell suspension at  $450 \times g$  for 10 min and resuspend the cells in 10 ml DPBS. Count cells and centrifuge again at  $450 \times g$  for 10 min at  $4^\circ\text{C}$ .
4. Add 10 ml DPBS with 2  $\mu$ M CFSE and mix it with the cell suspension (final CFSE concentration 1  $\mu$ M).
5. Incubate for 15 min at  $37^\circ\text{C}$  in a 50 ml tube.
6. Stop the reaction by adding 10 ml T cell medium and centrifuge for 7 min at  $450 \times g$ .
7. Resuspend the cells in 20 mL T cell medium and count the cells. Adjust the cells to  $1 \times 10^6$ /ml in T cell medium.

### Co-culture with BM-DC

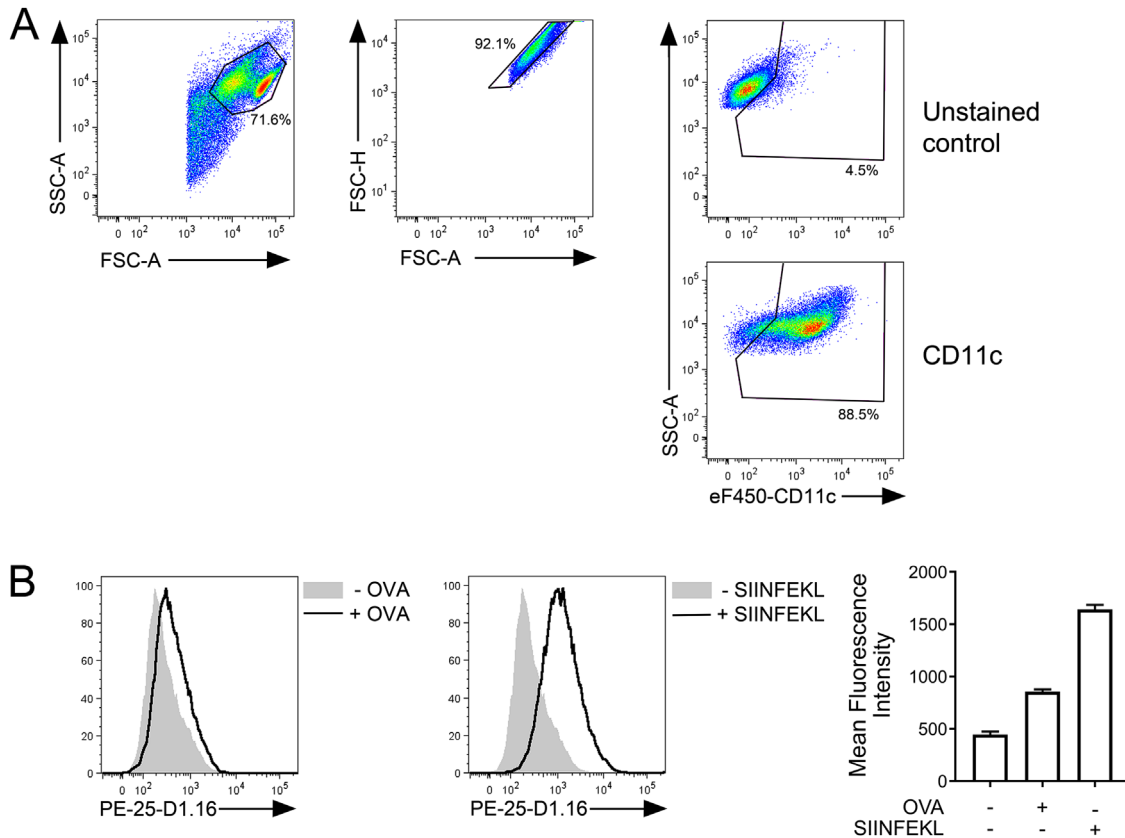
1. After 2 h of incubation with OVA or SIINFEKL, carefully remove the medium from the BM-DC
2. Add  $1 \times 10^5$  splenocytes (100  $\mu$ l) for co-culture.
3. After 1 day, add additional 200  $\mu$ l T cell medium to each well and incubate for 2 additional days.

### Flow cytometry

1. After 3 days, transfer all cells into FACS tubes and add 2 ml DPBS to each tube. Centrifuge at  $300 \times g$  for 5 min and discard the supernatant by decanting.
2. Prepare antibody mastermix  
DPBS containing mouse serum (1:100 diluted), fluorochrome-labeled antibodies against the surface antigens CD11b (dilution 1:800; final concentration 0.25  $\mu$ g/ml) and CD8 (dilution 1:400; final concentration 0.5  $\mu$ g/ml). also include single stained samples as controls for proper gating.
3. Add 50  $\mu$ l antibody master mix per tube
4. Incubate for 20 min at  $4^\circ\text{C}$
5. Wash the cells again with 2 ml DPBS and take the cells up in 100  $\mu$ l DPBS.
6. Analyze the cells by flow cytometry.

### 11.4 Data analysis

For all flow cytometry experiments, a BD LSR II Flow Cytometer was used and the data were analyzed using the FlowJo software. Spectrophotometrical analysis for ELISA was performed using a Microplate reader Infinite 200 PRO (Tecan).



**Figure 26.** Flow cytometric analysis of cross-presenting BM-DC using the 25-D1.16 antibody.

#### 11.4.1 Analysis of cross-presentation using the 25-D1.16 antibody

Cells were gated as indicated. 25-D1.16 staining is shown for OVA- or SIINFEKL-treated BM-DC (Fig. 26).

BM-DC were treated with OVA or SIINFEKL and stained with the 25-D1.16 antibody A) Exemplary gating of BM-DC. After elimination of doublets and debris, BM-DC are identified as CD11c<sup>+</sup> cells. B) 25-D1.16 staining of OVA- or SIINFEKL-treated BM-DC

#### 11.4.2 Analysis of OT-I T cell activation by ELISA

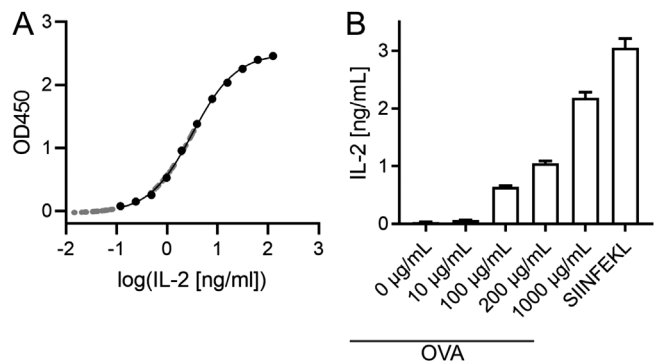
OD values were depicted by spectrometry as described above. Calculation of IL-2 concentrations was done as follows using GraphpadPrism

1. Subtract the OD of the blank from every other OD.
2. Transform the standard concentrations to logarithm of standard concentrations.
3. Plot the logarithm of concentration against the optical density (Fig. 27A).
4. Perform a sigmoidal curve fitting with interpolation of unknown values from the standard curve.
5. Transform the logarithm of concentrations to concentrations ( $x = 10^x$ )

#### 11.4.3 Analysis of OT-I T cell proliferation by flow cytometry

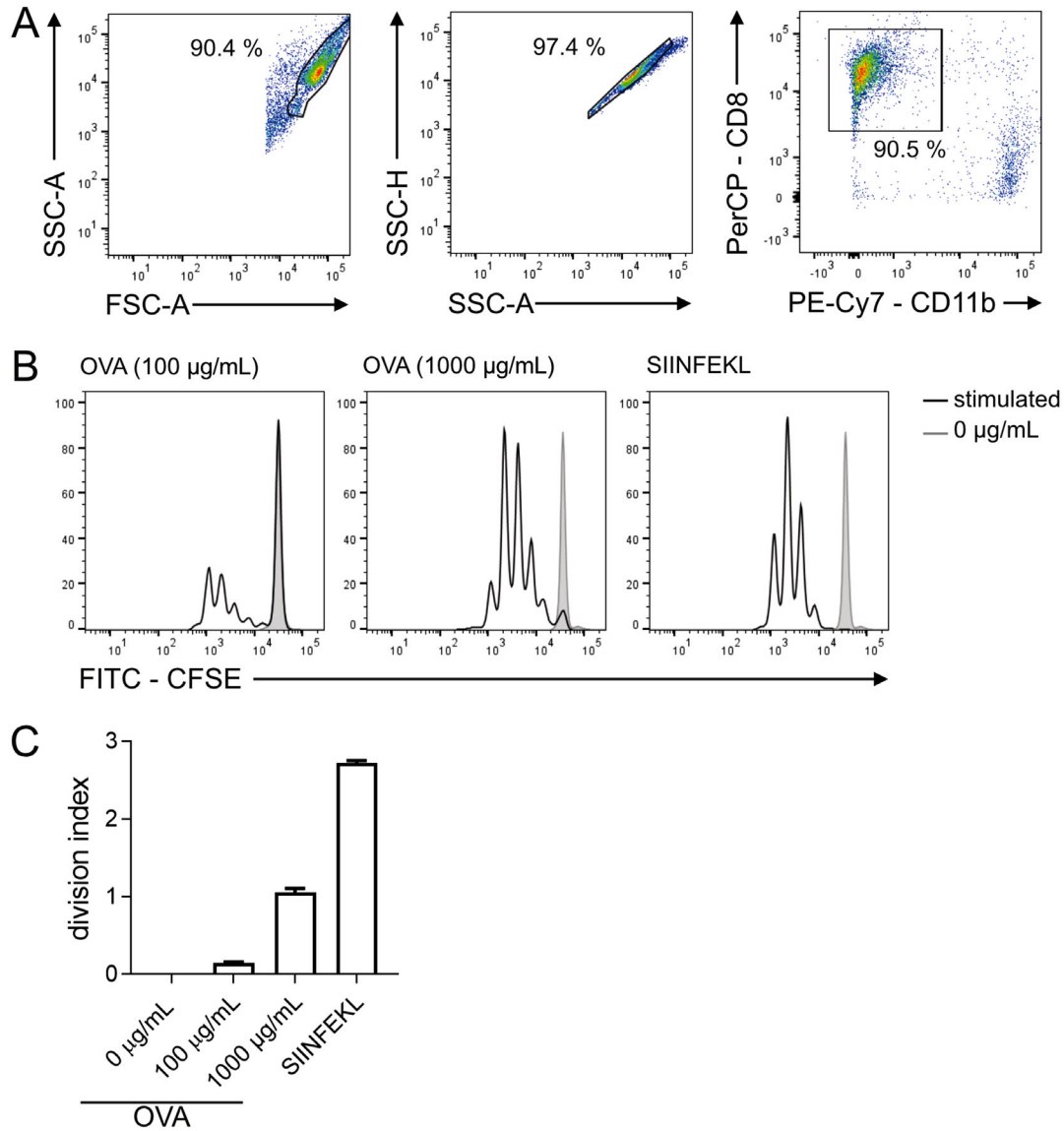
CFSE profiles were depicted by flow cytometry as described above and shown in Fig. 28.

There are several ways to quantify proliferation data. Often, the percentage of dividing cells is used for statistical analysis. However, such analysis does not include the amount of cell cycles individual cells went through. Therefore, calculation of



**Figure 27.** IL-2 secretion by OT-I T cells is dependent on OVA concentration used for stimulation of BM-DC. A) Exemplary sigmoidal standard curve. OD450 values of samples are shown in grey. B) IL-2 secretion by OT-I T cells co-cultivated with BM-DC stimulated with various concentrations of OVA or SIINFEKL peptide. Data are shown as mean  $\pm$  SEM.





**Figure 28.** Flow cytometric analysis of T cell proliferation induced by OVA-stimulated BM-DC.

the division index is the scientifically more appropriate way to quantify proliferation.

The division index is the average number of cell divisions that a cell in the original population has undergone. It can be calculated by the following formula:

$$\text{Division index} = \frac{\left[ 0 \cdot P_0 + 1 \cdot \frac{P_1}{2} + 2 \cdot \frac{P_2}{4} + 3 \cdot \frac{P_3}{8} + 4 \cdot \frac{P_4}{16} + 5 \cdot \frac{P_5}{32} + 6 \cdot \frac{P_6}{64} + 7 \cdot \frac{P_7}{128} + \dots \right]}{P_0 + \frac{P_1}{2} + \frac{P_2}{4} + \frac{P_3}{8} + \frac{P_4}{16} + \frac{P_5}{32} + \frac{P_6}{64} + \frac{P_7}{128} + \dots}$$

with P0 the percentage of cells undergoing no division, P1 the percentage of cells undergoing one cell division, P2 the percentage of cells undergoing 2 cell divisions,....

The division index can be calculated manually, setting gates for all populations with a specific number of cell cycles. Alterna-

tively, it can be calculated with the proliferation tool of the FlowJo software (see also 11.5.3.).

Co-culture of CFSE-loaded OT-I T cells with OVA-stimulated BM-DC. A) Exemplary gating of T cells. After elimination of doublets and debris, T cells are identified as CD8<sup>+</sup> and CD11b<sup>-</sup> cells. B) Proliferation profile of gated T cells. C) Division index of CD8<sup>+</sup> T cells co-cultured with BM-DC after OVA or SIINFEKL treatment. Data are shown as mean ± SEM.

### 11.5 Pitfalls

#### 11.5.1 Use of inhibitors/small molecules

When investigating the molecular mechanisms of cross-presentation, specific inhibitors/small molecules are often added

to the DC culture. However, to avoid a direct influence of such inhibitors on T cells, they need to be removed before the addition of T cells. Consequently, if the inhibition is reversible, their effect on DC would be lost during DC – T cell co-culture. To circumvent this problem, DC can be (mildly) fixed before the addition of T cells. To do this, DC are washed with DPBS after incubation with the antigen. Afterward, DC are fixed with 0.008% glutaraldehyde for exactly 3 min and washed twice with DPBS again. Then, T cells can be added as described above. It is of particular importance that the duration of this fixation step is identical for all samples.

### 11.5.2 Sensitivity of the 25-D1.16 antibody

Since the sensitivity of the 25-D1.16 antibody is not very high, the signal is not easy to distinguish from background signal and, in our hands, specific stainings are only possible using high amounts of antigen. For more sensitive assays with physiological antigen concentrations, T cell proliferation and IL-2 ELISAs should be performed. In addition, we obtained best results using a directly labeled antibody. In our hands, the use of a labeled secondary antibody also increased background staining, leading to a poor signal-to-noise ratio.

### 11.5.3 Calculation of the division index

FlowJo includes a function to calculate the division index. Therefore, the software identifies the individual peaks, which are used for its calculation, automatically. To our experience, the gates set by FlowJo do not always represent the correct populations. If you should decide to use this FlowJo feature, it is essential to check whether FlowJo set the gates correctly. If not, calculation of the division index using the formula described above might be the better choice.

## 11.6 Top tricks

The protocol provided here is based on the use of OT-I mice and MACS preparation of OT-I T cells. Alternatively, T cells can be isolated from OT-I/Rag2<sup>-/-</sup> mice. Since these cells lack endogenous B cells and CD4<sup>+</sup> T cells, the proportion of CD8<sup>+</sup> T cells is significantly higher and to our experience, MACS purification might be omitted.

Alternatively, when no OTI cells are available, the B3Z cell line can be used instead. After their activation, these OVA-specific hybridoma cells also secrete IL-2, which can be measured in the supernatants as described above. In addition, these cells express  $\beta$ -gal under control of the IL-2 promoter. Hence, their activation can also be measured colorimetrically after the addition of the chromogenic substrate CPRG.

## 12 In vivo analysis of killing efficacy by CD8<sup>+</sup> T cell responses

### 12.1 Introduction

Dendritic cells (DC) are important inducers of immune responses to pathogens. This includes the priming of CD8<sup>+</sup> cytotoxic T cells needed to kill virus-infected and malignant cells [1]. Therefore, it is of utmost importance to monitor the functional capacity of the induced T cell responses. This allows assessment of the efficacy and efficiency of immunogenic therapies using DC as inducer or mediator of anti-viral and/or antitumor responses without the need to directly induce tumor formation or infect animals [3]. The here described protocol allows for the simultaneous measurement of the killing efficacy of cytotoxic T cells to different peptides within a single animal. Thereby, the numbers of animals needed can be reduced and the T cells reactivity to different potential MHC I epitopes can be measured at the same time. The basis for the here presented protocol was published by Oehen et al., Quah et al., and Hermans et al. [184–186].

The principle of the assay is the differential labeling of the target cells and their loading with either different peptide concentrations and/or different peptides (different epitopes, irrelevant peptides). Therefore, splenocytes of a CD45.1-expressing mouse are used as target cells for CD45.2 mice. (Note: Any other congenic marker could also be used). We here use four different CFSE concentrations as well as four different CTV concentrations to label 16 different populations in total. Therefore, the CD45.1<sup>+</sup> splenocytes are equally divided into four aliquots. They are then labeled with the four different CFSE concentrations. Next, each of them is again divided in four aliquots, which is then labeled with a different CTV concentration. Afterwards, 4 × 4 = 16 populations are ready for peptide loading. Populations without peptide loading or which are loaded with an irrelevant peptide serve as internal standard and injection control. After loading, the cell populations will be mixed in equal numbers and injected into the immunized mice to test their immune reaction leading to the lysis of the injected target cells. After analysis of re-isolated splenocytes by flow cytometry (identifying the transferred cells by their CD45.1 expression), the efficacy of cell lysis is calculated by comparing the numbers of cells in a certain peptide-loaded population with the unloaded fraction (or the fraction loaded with an irrelevant peptide).

### 12.2 Materials

#### 12.2.1 Reagents

Necessary reagents are listed in Table 47.

#### 12.2.2 Equipment

Necessary equipment is listed in Tables 48 and 49.

**Table 47.** Reagents, chemicals and solutions

Reagent	Manufacturer	Ordering Number
Chemicals & Solutions		
Hank's Balanced Salt Solution without calcium and magnesium	Sigma	H6648
Dulbecco's Phosphate Buffered Saline without calcium and magnesium	Sigma	D8537
RPMI-1640	Sigma	R8758
Fetal Bovine Serum (FCS)	Sigma	F7524
CellTrace™ CFSE – Cell Proliferation Kit	Invitrogen	C34554
CellTrace™ Violet – Cell Proliferation Kit	Invitrogen	C34557
4',6-diamidino-2-phenylindole (DAPI)	Thermo Scientific	62247
Ammonium chloride (NH <sub>4</sub> Cl)	Carl Roth	K298.1
Potassium hydrogen carbonate (KHCO <sub>3</sub> )	Carl Roth	P748.1
SIINFEKL peptide (OVA <sub>257-264</sub> )	Invivogen	vac-sin
Antibodies		
Purified αFcyRIIB/III (2.4G2)	BioLegend	101302
Purified αFcyRIV (9E9)	BioLegend	149502
αCD45.1-PE	Biolegend	110708
αCD45.2-Alexa700	Biolegend	109822

**Table 48.** Necessary equipment

Equipment	Company	Purpose
Centrifuge 'Allegra X-15R'	Beckman-Coulter	Centrifugation of 50 ml tubes, 15 ml tubes and V-bottom plates
Neubauer counting chamber 0.100 mm; 0.0025 mm <sup>2</sup>	Superior Marienfeld	Cell counting
Corning storage bottle (#430518) and 0.22 μm sterile filter (#431118)	Corning	Sterile filtration and storage of solutions
Sterile bench 'Mars Safety Class 2'	Scanlaf	Performance of all aseptic procedures
LSR Fortessa SORP (#647800)	BD	Flow cytometric analysis of single-cell suspensions
96-well V-bottom plate (651 180)	Greiner bio-one	Sample preparation for flow cytometry
50 ml tubes (#352070)	Falcon	Centrifugation of cell suspensions
15 ml tubes (#188271)	Greiner bio-one	Centrifugation of cell suspensions
Serological pipettes (#606180)	Greiner bio-one	Pipetting
FACS tube (#352008)	Corning	Regular FACS tubes for acquisition of single-cell suspensions
100 μm filter for 50ml tubes (#542000)	Greiner bio-one	Isolation of single cells from tissues
40 μm filters for 50ml tubes (#542040)	Greiner bio-one	Generation of single-cell suspensions from lymphoid tissues by passive filtration
Pestles (#309658)	BD	Passage of organ material via 100 μm filters
6-well plates (#140675)	Thermo Scientific	Storage of organs & mechanical tissue disruption
Milli-Q Advantage A10	Millipore	On-demand provision of Millipore water (ddH <sub>2</sub> O)
Waterbath		

## 12.3 Step-by-step sample preparation

### 12.3.1 Preparation of stocks and solutions

For a list of reagents see Table 47.

#### DAPI

Dissolve DAPI 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) in ultrapure water to create a 1 mg/ml stock solution. Store the solution protected from light at 4°C. Dilute the stock solution 1:10,000 in FACS buffer (PBS+2% FCS) to create a working solution for DAPI staining of cells directly before acquisition at the flow cytometer.

#### FCS

Quickly thaw FCS at 37°C in a water bath. Once completely thawed, incubate for 15 min at 42°C in the water bath to destroy complement activity. Directly filter the warm FCS through a sterile 0.22 μm membrane (Corning #431118) into a sterile storage bottle (Corning #430518) and aliquot into 50 ml portions. Use aseptic techniques during the whole procedure. Aliquoted FCS must be stored at -20°C. Avoid freeze-thaw cycles.

#### FACS buffer

Add 2% FCS (v/v) to Phosphate buffered saline solution (PBS).

#### RPMI+2% FCS (CFSE & CTV Labeling buffer)

Add 2% FCS (v/v) to RPMI medium.

**Table 49.** Detailed configuration of the used BD LSR Fortessa

Laser line	Filter		Fluorochrome	Alternatives
	Long pass	Band pass		
355 nm	-	379/28	BUV395	-
	410LP	470/100	BUV496	DAPI, ZombieUV
	690LP	740/35	BUV737	-
405 nm	-	450/50	CTV	V450, e450, Pacific Blue, BV421
	505LP	525/50	V500	BV510, Pacific Orange
	545LP	585/42	BV570	-
	600LP	610/20	BV605	-
	630LP	670/50	BV650	-
	690LP	710/50	BV711	-
488 nm	-	488/10	FSC/SSC	-
	505LP	530/30	CFSE	FITC, GFP, A488
	685LP	710/50	PerCP-e710	PerCP-Cy5.5, PerCP
561 nm	-	586/15	PE	CMRA
	600LP	610/20	PE-Dazzle 594	PE-CF594
	635LP	670/30	PE-Cy5	PE-Dye649
	750LP	780/60	PE-Cy7	PE-Fire750
640 nm	-	670/14	A647	APC
	690LP	730/45	A700	-
	750LP	780/60	APC-Cy7	APC-Fire750, APC-H7, APC-e780

**CFSE stock solution**

Solubilize one CFSE vial (90 nmol) in 9.5  $\mu$ l water-free DMSO (final concentration 9.5 mM) and store small aliquots (e.g., 1  $\mu$ l) at  $-20^{\circ}\text{C}$ . Avoid freeze-thaw-cycles.

**CTV stock solution**

Solubilize one CTV vial (100 nmol) in 20  $\mu$ l water-free DMSO (final concentration 5 mM) and store small aliquots (e.g., 1  $\mu$ l) at  $-20^{\circ}\text{C}$ . Avoid freeze-thaw-cycles.

**Ammonium-Chloride-Potassium (ACK) lysis buffer for erythrocyte lysis**

Dissolve  $\text{NH}_4\text{Cl}$  and  $\text{KHCO}_3$  to reach final concentrations of 155 mM and 10 mM, respectively, in double distilled  $\text{H}_2\text{O}$ . Adjust the pH to 7.3. Autoclave the solution. Store at room temperature. After opening, do not use ACK lysis buffer longer than six weeks, since airborne  $\text{CO}_2$  in combination with carbonate lead to pH change of the buffer. Alternatively, cell-culture grade ACK lysis buffer is also commercially available.

**Generation of a SIINFEKL peptide (OVA<sub>257-264</sub>) stock solution**

Reconstitute lyophilized SIINFEKL peptide with sterile, endotoxin-free water to a stock concentration of 5mM under a cell culture hood. Store aliquots at  $-20^{\circ}\text{C}$ . Avoid freeze-thaw cycles.

**12.3.2 Immunization of mice**

Mice can be immunized with your preferred regimen priming cytotoxic  $\text{CD8}^+$  T cells. E.g., antibody targeting applying 100 ng

$\alpha$ DEC205-OVA in combination with 25  $\mu$ g poly(I:C) and 25  $\mu$ g  $\alpha$ CD40 antibody seven to eight days before the killing assay is a suitable immunization protocol. The here exemplary used antigen targeting approach allows for the specific delivery of antigens (in this case the model antigen ovalbumin, OVA) to cDC1 DC leading to a strong cytotoxic  $\text{CD8}^+$  T cell response. For more information see also Lehmann et al. and Dudziak et al [2, 3, 187]. Therefore, we inject 100 ng  $\alpha$ DEC205-OVA, 300 ng of  $\alpha$ DEC205-OVA, 100 ng Isotype-OVA, or 300 ng Isotype-OVA in combination with 25  $\mu$ g poly(I:C) and 25  $\mu$ g  $\alpha$ CD40 i. p. into C57Bl/6J mice.

**12.3.3 Preparation of splenocytes**

Within the article “Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues” [9] the harvesting of splenic material and the preparation of single-cell suspensions was described in detail in the section 1.1 Preparation of mouse single-cell suspensions.

In brief, following harvesting of spleen, tissues are transferred within a six well plate to a sterile working bench. All subsequent steps are performed under sterile conditions, using sterile buffers and equipment and aseptic techniques. Following mechanical tissue disruption via mincing with tweezers, cell suspensions are filtered via a 100 $\mu$ m cell strainer **without any previous digestion procedure**. Following centrifugation and erythrocyte lysis utilizing ACK lysis buffer, cell suspensions are passively filtered via a 40  $\mu$ m strainer and washed three times. The resulting cell suspension is ready for the labeling process.



**Table 50.** CFSE labeling scheme

Final CFSE concentration	Volume of 90 $\mu$ M CFSE solution to add to $10^6$ cells in 200 $\mu$ l medium
15 $\mu$ M	40 $\mu$ l
3 $\mu$ M	7.9 $\mu$ l
0.6 $\mu$ M	1.33 $\mu$ l
0 $\mu$ M	0 $\mu$ l

### 12.3.4 CFSE and CTV labeling of target cells

To identify the different cell populations after transfer into the immunized mice, CD45.1 donor cells are labeled with different concentrations of CTV and CFSE. First, splenic single-cell suspensions from a B6.SJL-Ptprca Pepcb/BoyJ (CD45.1 mice on a C57Bl/6 background).

1. Prewarm RPMI1640+2%FCS to 37°C.
2. Divide the single-cell suspension in four aliquots containing the same number of cells (e.g.,  $10^6$  cells).
3. Dilute 1  $\mu$ l of the CFSE stock solution (9.5 mM) with 104.6  $\mu$ l of PBS to reach a concentration of 90  $\mu$ M.
4. Centrifuge the prepared single-cell suspension aliquots (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
5. Resuspend the cell pellets in 200  $\mu$ l prewarmed RPMI1640+2% FCS per  $10^6$  cells. For a higher washing efficiency, we recommend using 15 ml tubes.
6. Add the respective amount of the PBS-diluted 90  $\mu$ M CFSE solution according to Table 50. It is important to mix immediately, to add the solution in the tubes one by one, and to start with the highest CFSE concentration. All provided volumes of RPMI+2% FCS and PBS diluted CFSE solution can be scaled up and down according to the cell count.
7. Incubate for 15 min @37°C in the waterbath (including the 0  $\mu$ M CFSE aliquot).
8. Fill up with cold RPMI1640+2% FCS to at least 10 ml and mix.  
Centrifuge the labeled cells (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
9. Resuspend the cells in 500  $\mu$ l prewarmed RPMI1640+2 %FCS.
10. Incubate for 10 min at 37°C in the waterbath (including the 0  $\mu$ M CFSE aliquot).
11. Centrifuge the labeled cells (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
12. Resuspend the cells in 420  $\mu$ l prewarmed RPMI1640+2 %FCS.
13. Now, divide each of the differentially CFSE-labeled populations in four 100  $\mu$ l aliquots (resulting in 16 different tubes).
14. In the next steps, these populations will be labeled with four different CTV concentrations (0, 0.5, 1.5, and 7.5  $\mu$ M). Therefore, each four tubes (from each CFSE labeling, namely 15  $\mu$ M, 3  $\mu$ M, 0.6  $\mu$ M, and 0  $\mu$ M) will be labeled with

**Table 51.** CTV labeling scheme

Final CTV concentration	Volume of 150 $\mu$ M CTV solution to add to 100 $\mu$ l medium
7.5 $\mu$ M	5.1 $\mu$ l
1.5 $\mu$ M	1 $\mu$ l
0.5 $\mu$ M	0.34 $\mu$ l
0 $\mu$ M	0 $\mu$ l

- the same concentration of CTV resulting in 16 differentially labeled cell aliquots.
15. Dilute 1  $\mu$ l of the CTV stock solution (5 mM) with 32.3  $\mu$ l of PBS to reach a concentration of 150  $\mu$ M.
  16. Centrifuge the prepared single-cell suspension aliquots (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
  17. Resuspend the cell pellets in 100  $\mu$ l prewarmed RPMI1640+2% FCS per aliquot. For a higher washing efficiency, we recommend using 15 ml tubes.
  18. Add the respective amount of the PBS-diluted 150  $\mu$ M CTV solution according to Table 51. It is important to mix immediately, to add the solution in the tubes one by one, and to start with the highest CTV concentration.
  19. Incubate for 15 min at 37°C in the waterbath (including the 0  $\mu$ M CTV aliquot).
  20. Fill up with cold RPMI1640+2% FCS to at least 10 ml.
  21. Centrifuge the labeled cells (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
  22. Resuspend the cells in 500  $\mu$ l prewarmed RPMI1640+2% FCS.
  23. Incubate for 10 min at 37°C in the waterbath (including the 0  $\mu$ M CTV aliquot).
  24. Centrifuge the labeled cells (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
  25. Resuspend the cells in each 100  $\mu$ l of prewarmed RPMI1640+2 %FCS.

### 12.3.5 Peptide loading of target cells and injection into the immunized mice

To investigate the killing of the target cells, the differentially labeled cell populations are pulsed with the MHC-I/CD8 peptides of interest. Here, we will describe an example for different concentrations of SIINFEKL peptide (the dominant CD8 epitope of Ovalbumin, OVA<sub>257-264</sub>), but the populations can also be loaded with different peptides and/or different concentrations.

1. Dilute the SIINFEKL stock solution (5 mM) to 1,250 nM (1:4,000) with prewarmed RPMI1640+2%FCS.
2. Prepare an additional 12.5 nM SIINFEKL dilution (1:100 from the 1,250 nM concentration) with prewarmed RPMI1640+2% FCS.
3. As example, we load the differentially labeled target cell populations with four different SIINFEKL concentrations, namely

**Table 52.** Overview of SIINFEKL peptide dose assignments to differentially CTV and CFSE labeled populations

	0 $\mu$ M CFSE	0.6 $\mu$ M CFSE	3 $\mu$ M CFSE	15 $\mu$ M CFSE
0 $\mu$ M CTV	625 nM SIINFEKL	625 nM SIINFEKL	unloaded	625 nM SIINFEKL
0.5 $\mu$ M CTV	160 nM SIINFEKL	160 nM SIINFEKL	2.5 nM SIINFEKL	160 nM SIINFEKL
1.5 $\mu$ M CTV	40 nM SIINFEKL	40 nM SIINFEKL	40 nM SIINFEKL	40 nM SIINFEKL
7.5 $\mu$ M CTV	2.5 nM SIINFEKL	2.5 nM SIINFEKL	160 nM SIINFEKL	2.5 nM SIINFEKL

625 nM, 160 nM, 40 nM, and 2.4 nM. Additionally, we prepare unloaded target cells (0 nM) as a reference. To minimize the effect of the CFSE/CTV labeling on the killing efficiency, we equally distribute the SIINFEKL concentrations to higher and lower labeled target cell populations and use replicates. Please see Table 52 for clarification.

- Add to 100  $\mu$ l of the respective target cell population the following amounts (Table 53) of the SIINFEKL dilutions and mix immediately:
- Incubate for 30 min at 37°C in the water bath.
- Fill up with RPMI1640+2% FCS to at least 10 ml and mix.
- Centrifuge (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
- Resuspend the cells in 1,000  $\mu$ l RPMI1640+2% FCS, fill up to at least 10 ml with RPMI1640+2% FCS and mix.
- Centrifuge (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
- Resuspend the cells in 1,000  $\mu$ l PBS and take an aliquot for cell counting before filling up to at least 10 ml with PBS.
- Determine the number of cells in each target cell population.
- Centrifuge (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
- Resuspend the cells in a small volume (e.g., 100  $\mu$ l) to mix all target cell populations in equal numbers (1:1:1:1:1 ... 1:1:1:1:1 ratio).
- Fill up to at least 10 ml with PBS.
- Centrifuge (e.g., 500–700  $\times$  g, 5–10 min, 4°C) and remove the supernatant completely.
- Resuspend the cells in room temperature (20°C) PBS for the injection into the immunized mice (about 2–10 $\times$ 10<sup>6</sup> cell in 50–100  $\mu$ l per mouse).
- Check a small aliquot of the cells at your flow cytometer to ensure their quality, labeling, and viability.

**Table 53.** Overview of added peptide amounts for peptide pulsing

Final SIINFEKL concentration	To add
625 nM SIINFEKL	100 $\mu$ l of the 1,250 nM dilution
160 nM SIINFEKL	74.4 $\mu$ l prewarmed RPMI160+2% FCS + 25.6 $\mu$ l of the 1,250 nM dilution
40 nM SIINFEKL	93.6 $\mu$ l prewarmed RPMI160+2% FCS + 6.4 $\mu$ l of the 1,250 nM dilution
2.5 nM SIINFEKL	60 $\mu$ l prewarmed RPMI160+2% FCS + 40 $\mu$ l of the 12.5 nM dilution
unloaded	100 $\mu$ l prewarmed RPMI1640+2% FCS

- Inject the mixed target cell populations intravenously into immunized mice.

### 12.3.6 Analysis of killing efficacy

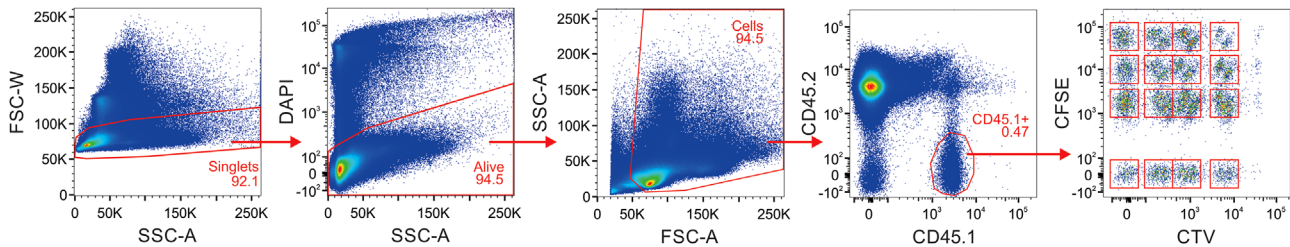
To investigate the killing of the target cells, splenic single-cell suspensions will be generated and analyzed for their content of injected target cell populations. Splenic single-cell suspensions will be generated as described within article **Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues** [9] in section 1.1 **Preparation of mouse single-cell suspensions** and as summarized under point 12.3.3 within this section. For effective killing, spleens of mice are harvested 8 to 16 h after target cell transfer.

In brief, following harvesting of spleen, tissues are transferred into a 6-well plate. Subsequent to mechanical tissue disruption via mincing with tweezers, cell suspensions are filtered via a 100  $\mu$ m cell strainer **without any previous digestion procedure**. Following centrifugation and erythrocyte lysis utilizing ACK lysis buffer, cell suspensions are passively filtered via a 40  $\mu$ m strainer and washed three times. The resulting cell suspension is ready for the labeling process.

- Following the preparation of single-cell suspensions, transfer 10 $\times$ 10<sup>6</sup> cells per spleen to a 96-well V-bottom plate;
- Before centrifugation of the samples, prepare the Fc receptor-blocking solution containing purified  $\alpha$ Fc $\gamma$ RIIB/III (clone 2.4G2; stock 0.5 mg/ml) and  $\alpha$ Fc $\gamma$ RIV (clone 9E9; stock 0.5 mg/ml) in a dilution of 1:400;
- Centrifuge the sample containing 96-well V-bottom plates at 700  $\times$  g for 5 min at 4°C. All following centrifugations are carried out using these settings;
- Resuspend cells in 50  $\mu$ l of the Fc receptor-blocking solution;
- Incubate the samples for 5 min at 4°C.
- In the meantime, prepare the antibody staining cocktail. Therefore, use 50  $\mu$ l of FACS buffer per sample and add the antibodies listed in Table 54 in the indicated dilution; Please note that antibody dilutions are dependent on the

**Table 54.** Antibody staining mix for flow cytometry

Fluorophore/ Labeling	Antigen	Clone	#Catalog	Company	Dilution
PE	CD45.1	A20	110708	Biologend	1:1,000
Alexa700	CD45.2	104	109822	Biologend	1:100



**Figure 29.** Exemplary gating strategy for the transferred cells in the immunized mice.  $2 \times 10^6$  target cells were transferred intravenously into immunized C57Bl/6J mice. 16 h later, splenic single-cell suspensions were prepared and  $10 \times 10^6$  splenocytes were stained with CD45.1-PE (1:1,000) and CD45.2-Alexa700 (1:100). After washing, cells were analyzed using a BD LSR Fortessa SORP flow cytometer. First, doublet cells (ratio SSCA/FSCW), dead cells (DAPI positive) as well as debris were excluded. Next, transferred cells were identified as CD45.1<sup>+</sup> and CD45.2<sup>-</sup>. From these cells, the 16 differentially labeled transferred target cell populations were identified according to their CFSE and CTV fluorescence. Here, we present one exemplary gating strategy after transfer of the CD45.1 target cell population mixture into a C57Bl/6J mouse. The data was acquired on a BD LSR Fortessa SORP flow cytometer and evaluated with FlowJo v10.8 software.

- flow cytometer and its setup; Suggested antibody dilutions were optimized for a BD LSR Fortessa equipped with 355 nm, 405 nm, 488 nm, 561 nm, and 640 nm laser lines;
7. Centrifuge the sample containing 96-well V-bottom plates at  $700 \times g$  for 5 min at 4°C;
  8. Discard the supernatant, keep the plate up-side down and dip on a paper towel;
  9. Resuspend each sample in 50  $\mu$ l of primary antibody staining mix and incubate for 30 min at 4°C;
  10. Fill up with 100  $\mu$ l FACS buffer per well and centrifuge;
  11. Discard the supernatant, keep the plate up-side down and dip on a paper towel;
  12. Resuspend each sample in 160  $\mu$ l FACS buffer;
  13. Centrifuge at  $700 \times g$  and 4°C for 5 min;
  14. Discard the supernatant, keep the plate up-side down and dip on a paper towel;
  15. Resuspend each sample in 160  $\mu$ l FACS buffer;
  16. Centrifuge at  $700 \times g$  and 4°C for 5 min;
  17. Resuspend each sample in 100  $\mu$ l of FACS buffer;
  18. Samples are ready for acquisition at a flow cytometer;
  19. Add 180  $\mu$ l of FACS buffer containing DAPI in a dilution of 1:10,000 directly before acquisition;
  20. After performing cytometer setup, acquire data at an appropriate flow cytometer;

## 12.4 Data analysis

Data of  $2.5 \times 10^6$  cells was acquired utilizing a BD LSR Fortessa SORP flow cytometer (configuration see Table 49) and analyzed by the commercial software FlowJo v10.8 (BD). After exclusion of doublets, debris, and dead cells, transferred cells were identified as CD45.1<sup>+</sup> CD45.2<sup>-</sup> (see Fig. 29). These cells were analyzed for their CFSE and CTV fluorescence and the number of cells within the 25 different clusters were extracted. The number of not peptide loaded cells served as control for injection efficiency as well as unspecific killing. An exemplary gating is displayed in Fig. 29. Lysis for the different SIINFEKL concentrations is calculated as percentage of non-peptide-loaded cells (example data are presented in Fig. 30).

## 12.5 Pitfalls

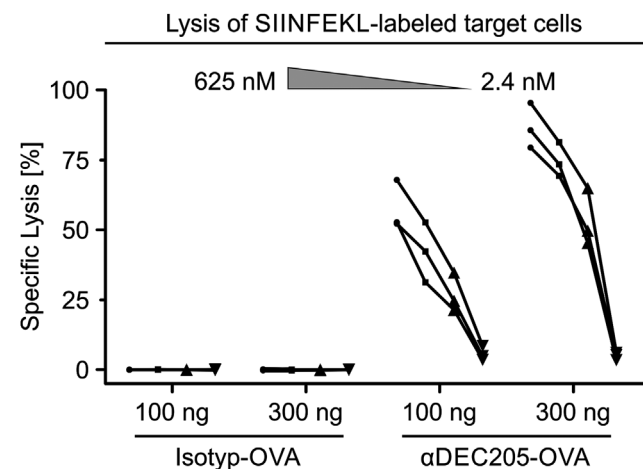
**Problem:** The labeling of the target cell population is not uniform enough leading to difficulties in the separation after transfer.

**Potential solution:**

Ensure the direct mixing after adding the CFSE or CTV dye to the cells.

Adapt the PMT voltages to maximize the separation of the different populations.

Re-titrate the concentration of CTV and/or CFSE to optimize it for your flow cytometer.



**Figure 30.** Exemplary data for lysis of target cells after immunization. Eight weeks old, female C57Bl/6J mice were immunized with either 100 ng or 300 ng of the targeting antibody  $\alpha$ DEC205-OVA or the respective Isotype control (Isotype-OVA) in combination with 25  $\mu$ g poly(I:C) and 25  $\mu$ g  $\alpha$ CD40 antibody intraperitoneally. Three weeks later,  $2 \times 10^6$  target cells were transferred intravenously into immunized C57Bl/6J mice. Another 16 h later, splenic single-cell suspensions were prepared and  $10 \times 10^6$  splenocytes were stained with CD45.1-PE (1:1,000) and CD45.2-Alexa700 (1:100) and analyzed as described in Fig. 29. The cell numbers in each transferred population were evaluated with FlowJo v10.8 software and the lysis of the target cells was calculated using a classical Excel sheet by dividing the number of SIINFEKL labeled cells by the number of unlabeled cells re-isolated from each mouse. Presented is the median specific depletion of the target cells of

**Problem: No killing of target cells.****Potential solution:**

Ensure that the target cells have the right haplotype for the used peptides. E.g., SIINFEKL is dependent on the H-2K<sup>b</sup> molecule. Therefore, the target cells as well as the immunized mice need to express H-2K<sup>b</sup> alloantigen. To ensure labeling of the target cells with SIINFEKL, an  $\alpha$ SIINFEKL bound to H-2K<sup>b</sup> antibody (clone 25-D1.16, available from Biologend, e.g., 141606 for an APC conjugate), might be used.

**12.6 Top tricks**

In this protocol, we use two different dyes to label 16 to 25 different populations. To enhance the value of the assay, a third fluorescence dye can be added (leading to the distinction of 64 to 125 different target populations).

In the example data provided here, we only used one well-defined peptide, namely SIINFEKL, from Ovalbumin in different concentrations. This protocol can be easily adapted to: 1) loading of different peptides, 2) more peptide concentrations (to assess avidity), or even to peptide libraries.

Furthermore, this assay can be applied to evaluate memory-recall-responses or responses in different organs, such as lung, liver, etc. If assessing memory CD8<sup>+</sup> T cell-mediated killing responses, we suggest to evaluate the optimal time point after target cell transfer as a memory re-call might need longer than the usual 8 to 16 hours.

The assay can also be extended to assess NKT cell-mediated killing, e.g., loading target cells with lipids of interest, such as  $\alpha$ GalCer.

As the methods focus solely on the number of killed cells, it can be extended to tumor cell killing and NK-mediated killing by using another target cell mixture. We recommend to always add spleen cells as one population as they can serve as optimal control for injection efficiency (as no-target cell population) and unspecific loss.

**13 Measuring naïve T cell responses induced by human primary DC****13.1 Introduction**

Dendritic cells (DC) are the main regulators of immunity due to their capacity to induce naïve T cell responses [188, 189]. In mice, DC subpopulations show clear differences in their capacity to polarize naïve CD4<sup>+</sup> T cells as well as to cross-present antigens to CD8<sup>+</sup> T cells *in vivo* [2, 190, 191]. Here, cDC1 mainly induce Th1 CD4<sup>+</sup> T cell responses and are the main cross-presenting DC subpopulations, while cDC2 preferentially induce Th2 as well as Th17 CD4<sup>+</sup> T cell responses [2, 190–193]. While all human DC subpopulations seem to be able to cross-present antigens *in vitro*, results on polarization of CD4<sup>+</sup> T cells are quite heterogeneous [194–199].

We here describe a protocol to perform mixed leukocyte responses (MLR) with cell-sorted human primary blood DC and naïve CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells by negative enrichment. DC can be stimulated with TLR ligands prior to the co-culture in order to analyse the effect of TLR-induced activation on the capacity of the DC to induce proliferation as well as polarization of the T cells. Here, the TLR ligand R848 was used as it is able to induce IL-12 secretion by human DC subpopulations, which should induce Th1 polarization as well as IFN $\gamma$  secretion by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, respectively. However, also ligands for other pattern recognition receptors might be used, which can influence the secretion of cytokines and, thereby, the phenotype of the induced T cell response [188].

**13.2 Materials****13.2.1 Reagents**

A complete list of reagents is provided in Table 55. Further, detailed protocols for cell-sorted primary DC as well as PBMCs of a HLA-mismatched blood donor are detailed in [156] sections 3 “Cell sorting of primary human DC” and section 1.1 “Isolation of mononuclear cells from peripheral blood”, respectively.

**13.2.2 Equipment**

Necessary equipment is listed in Tables 56 and 57.

**13.3 Step-by-step sample preparation****13.3.1 Preparation of stocks and solutions****DC/T cell medium**

Add 10% human sera type AB (v/v), 1% non-essential amino acids, 1% Penicillin-Streptomycin (v/v), 1% L-Glutamine (v/v), and 1% sodium pyruvate (v/v) to 500 ml RPMI-1640.

**R848**

Prepare a 1 mg/ml stock solution of R848 by adding 500  $\mu$ l of endotoxin-free water to 500  $\mu$ g of R848. Vortex the solution until it is completely dissolved. Prepare aliquots and store them at  $-20^{\circ}\text{C}$ .

**EasySep Buffer**

Add 2% human sera type AB and 1 mM EDTA to 500 ml PBS.

**CFSE**

To prepare a 10 mM stock solution of CFSE, add 9  $\mu$ l of DMSO to the CellTrace<sup>TM</sup> CFSE dye and mix well. Make 1  $\mu$ l aliquots and store them at  $-20^{\circ}\text{C}$ .



**Table 55.** List of needed reagents for the preparation of single cells from human lymphoid tissues

Reagent	Manufacturer	Ordering number
<b>Antibodies</b>		
$\alpha$ CD3 BUV395 (UCHT1)	BD Biosciences	563548
$\alpha$ CD4 BV510 (OKT4)	BioLegend	317444
$\alpha$ CD8 BUV737 (SK1)	BD Biosciences	564629
$\alpha$ CD25 PerCP/Cy5.5 (M-A251)	BioLegend	356112
$\alpha$ IL-2 PE/Dazzle594 (MQ1-17H12)	BioLegend	500344
$\alpha$ IL-4 A647 (8D4-8)	BioLegend	500712
$\alpha$ IL-17A BV605 (BL168)	BioLegend	512326
$\alpha$ IL-10 PE (JES3-9D7)	BioLegend	501403
$\alpha$ IFN $\gamma$ BV421 (4S.B3)	BioLegend	502532
$\alpha$ TNF $\alpha$ PE/Cy7 (MAb11)	BioLegend	502930
$\alpha$ Granzyme B A647 (GB11)	BioLegend	515406
$\alpha$ Perforin PE (B-D48)	BioLegend	353304
Mouse IgG1 isotype control PerCP/Cy5.5 (MOPC-21)	BioLegend	400150
Rat IgG2a isotype control PE/Dazzle594 (RTK2758)	BioLegend	400558
Mouse IgG1 isotype control A647 (MOPC-21)	BioLegend	400136
Mouse IgG1 isotype control BV605 (MOPC-21)	BioLegend	400162
Rat IgG1 isotype control PE (G0114F7)	BioLegend	401906
Mouse IgG1 isotype control BV421 (MOPC-21)	BioLegend	400158
Mouse IgG1 Isotype control PE/Cy7 (MOPC-21)	BioLegend	400126
Mouse IgG1 isotype control PE (MOPC-21)	BioLegend	400140
<b>Chemicals &amp; Solutions</b>		
Dulbecco's Phosphate Buffered Saline without calcium and magnesium	Sigma-Aldrich	D8537
RPMI-1640 Medium	Sigma-Aldrich	R8758
Human Serum type AB (male)	Sigma-Aldrich	H4522
UltraPure™ 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020
Trypan blue	Sigma-Aldrich	T6146
R848 (Resiquimod)	Invivogen	tlr-r848
4',6-diamidino-2-phenylindole (DAPI)	Thermo Scientific	62247
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific	11140050
Penicillin-Streptomycin	Sigma-Aldrich	P4333
L-Glutamine	Sigma-Aldrich	G7513
Sodium Pyruvate	Sigma-Aldrich	S8636
Phorbol myristate acetate (PMA)	Invivogen	tlrl-pma
Ionomycin	Invivogen	inh-ion
Brefeldin A Solution (1,000X)	BioLegend	420601
<b>Kits</b>		
EasySep™ Human Naïve CD4 <sup>+</sup> T Cell Isolation Kit II	Stemcell Technologies	17555
EasySep™ Human Naïve CD8 <sup>+</sup> T Cell Isolation Kit II	Stemcell Technologies	17968
CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry	Thermo Fisher Scientific	C34554
Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation	Thermo Fisher Scientific	11131D
Zombie UV™ Fixable Viability Kit	BioLegend	423108
Cytofix/Cytoperm™ Fixation/Permeabilization Kit	BD Biosciences	554714

**Phorbol myristate acetate (PMA)**

Prepare a 5 mg/ml stock solution of PMA by adding 1 ml of DMSO to 5 mg of PMA. Vortex until PMA is completely dissolved. Prepare aliquots and store them at  $-20^{\circ}\text{C}$ .

**Ionomycin**

Prepare a 10 mg/ml stock solution of Ionomycin by adding 100  $\mu\text{l}$  of DMSO to 1 mg of Ionomycin. Vortex until Ionomycin is completely dissolved. Prepare aliquots and store them at  $-20^{\circ}\text{C}$ .

**Restimulation medium**

Add 1  $\mu\text{g}/\text{ml}$  PMA, 5  $\mu\text{g}/\text{ml}$  Ionomycin as well as 1% Brefeldin A solution (1000x) to DC/T cell medium.

**Zombie UV™ Fixable Viability Dye**

Add 100  $\mu\text{l}$  of DMSO to one vial of Zombie UV™ Dye and pipet up and down until it is completely dissolved. Prepare 10  $\mu\text{l}$  aliquots and store at  $-80^{\circ}\text{C}$ .

**Table 56.** Necessary equipment

Equipment	Company	Purpose
Centrifuge “Allegra X-15R”	Beckman-Coulter	Centrifugation of 50 ml tubes, 15 ml tubes and V-bottom plates
Incubator “HERAEUS BBD6220”	Thermo Scientific	Cabinet style incubator with 5% CO <sub>2</sub> and 96% relative humidity for the lymphoid tissue digestion
LSR Fortessa (#647800)	BD	Flow cytometric analysis of single-cell suspensions
Neubauer chamber 0.100 mm; 0.0025 mm <sup>2</sup>	Superior Marienfeld	Cell counting
Sterile bench “Mars Safety Class 2”	Scanlaf	Performance of all aseptic procedures
96 Well Cell Culture Plate (V-bottom) (#651 180)	Greiner bio-one	Plates for the stimulation of sorted DC in sterile conditions
Clear Flat-Bottom Immuno Non-sterile 96-Well Plates (#442404)	Thermo Scientific	Performance of the LDH assay
50 ml tubes (#352070)	Falcon	Preparation of 1 x Wash buffer (LEGENDplex assay)
14 ml tubes, round bottom, (#187262)	Greiner bio-one	Isolation of naïve CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells
Serological pipettes (#606180)	Greiner bio-one	Pipetting
“The Big Easy” EasySep™ Magnet (#18001)	Stemcell Technologies	Isolation of naïve CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells

**FACS buffer**

Add 2% human serum type AB (v/v) to 500 ml of Phosphate buffered saline solution (PBS).

**1 x Perm/Wash Buffer**

Dilute the 10× Perm/Wash Buffer (Cytotfix/Cytoperm™ Fixation/Permeabilization Kit) 1:10 with ddH<sub>2</sub>O and mix well by inverting.

**13.3.2 Measuring naïve T cell responses using mixed leukocyte reactions with primary DC****13.3.2.1 Stimulation of primary DC with TLR ligands.**

1. Isolate primary human cDC1, cDC2, pDC, and monocytes as described in section 3 “Cell sorting of primary human DC” in [156]. Resuspend the isolated DC in DC medium to a final concentration of  $2 \times 10^5$  cells/ml.

**Table 57.** Detailed configuration of the used BD LSR Fortessa

Laser line	Filter		Fluorochrome	Alternatives
	Long pass	Band pass		
355 nm	-	379/28	BUV395	-
	410LP	470/100	DAPI	BUV496, ZombieUV
	690LP	740/35	BUV737	-
405 nm	-	450/50	BV421	V450, e450, Pacific Blue, CTV
	505LP	525/50	BV510	V500, Pacific Orange
	545LP	585/42	BV570	-
	600LP	610/20	BV605	-
	630LP	670/50	BV650	-
	690LP	710/50	BV711	-
	488 nm	-	488/10	FSC/SSC
505LP	505LP	530/30	FITC	A488, GFP, CFSE
	685LP	710/50	PerCP-Cy5.5	PerCP-e710, PerCP
561 nm	-	586/15	PE	Cy3, CMRA
	600LP	610/20	PE-Dazzle 594	PE-CF594
	635LP	670/30	PE-Cy5	PE-Dye649
	750LP	780/60	PE-Cy7	PE-Fire750
640 nm	-	670/14	APC	A647
	690LP	730/45	A700	-
	750LP	780/60	APC-Fire750	APC-Cy7, APC-H7, APC-e780

2. Seed 10,000 (50  $\mu$ l) primary cDC1, cDC2, pDC, or monocytes into the wells of 96-well plate (U-bottom).
3. Prepare a 10  $\mu$ g/ml solution of R848 by diluting R848 stock solution 1:100 in DC/T cell medium.
4. Add 50  $\mu$ l of diluted R848 (final concentration of 5  $\mu$ g/ml) or 50  $\mu$ l of DC/T cell medium to the DC or monocytes in the 96-well plate.
5. Incubate for 3 h at 37°C in the incubator to induce activation or not in the cultured DC.
6. During incubation, proceed with isolation of naïve CD4<sup>+</sup> T cells (13.3.2.2.1.) or naïve CD8<sup>+</sup> T cells (13.3.2.2.2.).

### 13.3.2.2 Isolation of naïve T cells.

#### 13.3.2.2.1 Isolation of Naïve CD4<sup>+</sup> T cells.

7. Transfer  $2 \times 10^8$  PBMCs of a HLA-mismatched donor into 14 ml round bottom tubes. Add EasySep buffer to adjust to a final concentration of  $5 \times 10^7$  cells/ml.
8. Add 200  $\mu$ l (50  $\mu$ l/ml cell suspension) EasySep™ Human Naïve CD4<sup>+</sup> T Cell Isolation Cocktail II (Component #17555C) to the cell suspension, mix well and incubate for 5 min at RT.
9. Vortex the EasySep™ Dextran RapidSpheres™ (Component #50103) for at least 30 s. Then, add 200  $\mu$ l (50  $\mu$ l/ml cell suspension) of the EasySep™ Dextran RapidSpheres™ to the cell suspension and fill up to 10 ml using EasySep buffer.
10. Remove the lid of the tube and place it into “The Big Easy” EasySep™ Magnet and incubate it at RT for 3 min.
11. Leave the tube inside the magnet, while pouring off the cell suspension into a new 14 ml tube.
12. Remove the tube from the magnet and throw it away. Place the new 14 ml tube (step 11) without lid into the magnet and incubate again for 3 min at RT.
13. Leave the tube inside the magnet, while pouring off the cell suspension into a new 14 ml tube.
14. The new 14 ml tube (Step 13) contains the isolated naïve CD4<sup>+</sup> T cells. Count the cells using a Neubauer chamber.

#### 13.3.2.2.2 Isolation of naïve CD8<sup>+</sup> T cells.

15. Transfer  $2 \times 10^8$  PBMCs of a HLA-mismatched donor into 14 ml round bottom tubes. Add EasySep buffer to adjust to a final concentration of  $5 \times 10^7$  cells/ml.
16. Add 200  $\mu$ l (50  $\mu$ l/ml cell suspension) of EasySep Human Naïve CD8<sup>+</sup> T Cell Isolation Cocktail (Component #19258C), mix well, and incubate for 5 min at RT.
17. Vortex the EasySep™ Dextran RapidSpheres™ (Component #50103) for at least 30 s. Then, add 200  $\mu$ l (50  $\mu$ l/ml cell suspension) of the EasySep™ Dextran RapidSpheres™ to the cell suspension and fill up to 10 ml using EasySep buffer.
18. Remove the lid of the tube and place it into “The Big Easy” EasySep™ Magnet and incubate it at RT for 3 min.

19. Leave the tube inside the magnet, while pouring off the cell suspension into a new 14 ml tube.
20. Remove the tube from the magnet and throw it away. Place the new 14 ml tube (step 19) without lid into the magnet and incubate again for 3 min at RT.
21. Leave the tube inside the magnet, while pouring off the cell suspension into a new 14 ml tube.
22. The new 14 ml tube (Step 21) contains the isolated naïve CD8<sup>+</sup> T cells. Count the cells using a Neubauer chamber.

### 13.3.2.3 Labeling of isolated naïve T cells with CFSE.

23. Add 199  $\mu$ l of PBS to an aliquot of the 10 mM CFSE stock solution (dilutes the stock to a concentration of 50  $\mu$ M).
24. Resuspend  $5 \times 10^6$  naïve CD4<sup>+</sup> (13.2.2.1.) or CD8<sup>+</sup> (13.2.2.2.) T cells in 90  $\mu$ l of pre-warmed RPMI-1640 after washing the cells with PBS twice. Add 10  $\mu$ l (1:10 dilution; final concentration of 5  $\mu$ M) of the diluted CFSE (step 23) to the T cells. Mix well and incubate at 37°C for 20 min.
25. Fill up to 10 ml with ice-cold DC/T cell medium to stop labeling of the T cells. Centrifuge for 5 min with  $520 \times g$  at 4°C.
26. Wash the cells with 10 ml of DC/T cell medium and count the cells using a Neubauer chamber. Adjust the cell concentration to  $5 \times 10^5$  cells/ml in DC/T cell medium.

### 13.3.2.4 Performing mixed leukocyte reactions.

27. Pre-warm DC/T cell medium as well as the T cells to 37°C.
28. Remove the 96-well plate after the 3 h incubation time from the incubator (step 5). Centrifuge for 5 min with  $520 \times g$  at RT. Carefully remove the medium from each well using a pipette.
29. Resuspend the DC in 100  $\mu$ l of pre-warmed DC/T cell medium and centrifuge for 5 min with  $520 \times g$  at RT. Carefully remove the medium from each well using a pipette.
30. If you want to analyze naïve CD4<sup>+</sup> T cell responses, add 200  $\mu$ l (100,000 cells) of the pre-warmed CFSE-labeled naïve CD4<sup>+</sup> T cells to each well. If you want to analyze naïve CD8<sup>+</sup> T cell responses, add 200  $\mu$ l (100,000 cells) of the pre-warmed CFSE-labeled naïve CD8<sup>+</sup> T cells to each well.
31. Additionally, add 200  $\mu$ l of the T cells to six wells of the plate. Add 2.5  $\mu$ l  $\alpha$ CD3/CD28-coated Dynabeads (corresponding to 100,000 beads) to three of the wells as positive control, while the other three wells serve as negative control.
32. Co-culture the DC and T cells (corresponds to a ratio of 1:10) for 6 days at 37°C in the incubator (dependent on the aim of the analysis, also shorter periods of co-culture are possible; see 13.6 Top tricks). During the culture, regularly check the plate for consumed media (color change to orange-yellow). If the medium is consumed, centrifuge the plate for 5 min with  $300 \times g$  at RT. Remove 100  $\mu$ l of the medium from the wells and add 100  $\mu$ l fresh pre-warmed DC/T cell medium. Gently resuspend the well and proceed the co-culture in the incubator.

**Table 58.** Extracellular antibody staining panel for MLR with naïve CD4<sup>+</sup> T cells

Fluorophore/ Labeling	Antigen	Clone	#Catalog	Company	Dilution
BUV395	CD3	UCHT1	563548	BD Bio- sciences	1:100
BV510	CD4	OKT4	317444	BioLegend	1:100
PerCP/Cy5.5	CD25	M-A251	356112	BioLegend	1:200

**Table 59.** Extracellular antibody staining panel for MLR with naïve CD8<sup>+</sup> T cells

Fluorophore/ Labeling	Antigen	Clone	#Catalog	Company	Dilution
BUV395	CD3	UCHT1	563548	BD Bio- sciences	1:100
BUV737	CD8	SK1	564629	BD Bio- sciences	1:100
PerCP/Cy5.5	CD25	M-A251	356112	BioLegend	1:200

### 13.3.2.5 Flow cytometric analysis of T cell proliferation, activation, and polarization.

33. For each well, prepare 20 µl of restimulation medium by diluting PMA (1:5,000), Ionomycin (1:2,000), and Brefeldin A (1:100) in DC/T cell medium. In case you prepare less than 2,500 µl, perform pre-dilutions of PMA and Ionomycin. Pre-warm the restimulation medium to 37°C.
34. After the incubation time, add 20 µl of restimulation medium to each well to reach a final concentration of 100 ng/ml PMA, 500 ng/ml Ionomycin, and 0.1% Brefeldin A. Resuspend the wells and incubate for 6 h at 37°C.
35. Transfer the content of each well to a 96-well V-bottom plate. Centrifuge the plate for 5 min with 520 × g at 4°C. Remove the supernatant.
36. Rinse the U-bottom plate with PBS to collect cells that remained in the wells and use it to resuspend the cells in the corresponding well on the 96-well V-bottom plate.
37. Centrifuge the plate for 5 min with 520 × g at 4°C. Remove the supernatant and resuspend the cells in PBS. Centrifuge the plate for 5 min with 520 × g at 4°C.
38. Remove the supernatant and resuspend the cells in 50 µl PBS + Zombie UV<sup>TM</sup> Fixable Viability Dye (1:200). Incubate on ice for 15 min in the dark.
39. Add 100 µl FACS buffer and centrifuge for 5 min with 520 × g at 4°C.
40. Remove the supernatant and resuspend the cell pellet in 100 µl FACS buffer. Centrifuge for 5 min with 520 × g at 4°C. During centrifugation, prepare a master mix (50 µl/sample) for staining CD4<sup>+</sup> T cells (Table 58) or CD8<sup>+</sup> T cells (Table 59) dependent on which cells were used for the MLR. Dilute the antibodies according to Table 58 or Table 59 in FACS buffer. Further, prepare one sample with mouse IgG1 isotype control PerCP/Cy5.5 instead of αCD25 PerCP/Cy5.5.

**Table 60.** Intracellular antibody staining panel for MLR with naïve CD4<sup>+</sup> T cells

Fluorophore/ Labeling	Antigen	Clone	#Catalog	Company	Dilution
PE/Dazzle594	IL-2	MQ1- 17H12	500344	BioLegend	1:200
A647	IL-4	8D4-8	500712	BioLegend	1:100
BV605	IL-17A	BL168	512326	BioLegend	1:100
PE	IL-10	JES3- 9D7	501403	BioLegend	1:50
BV421	IFN <sub>γ</sub>	4S.B3	502532	BioLegend	1:100
PE/Cy7	TNFα	MAb11	502930	BioLegend	1:200

**Table 61.** Intracellular antibody staining panel for MLR with naïve CD8<sup>+</sup> T cells

Fluorophore/ Labeling	Antigen	Clone	#Catalog	Company	Dilution
PE/Dazzle594	IL-2	MQ1- 17H12	500344	BioLegend	1:200
A647	Granz- yme B	GB11	515406	BioLegend	1:100
BV605	IL-17A	BL168	512326	BioLegend	1:100
PE	Perforin	B-D48	353304	BioLegend	1:50
BV421	IFN <sub>γ</sub>	4S.B3	502532	BioLegend	1:100
PE/Cy7	TNFα	MAb11	502930	BioLegend	1:200

41. Remove the supernatant and resuspend all samples (with DC) in 50 µl of the prepared master mix as well as two wells of the positive (with CD3/CD28 Dynabeads) and negative (T cells only) controls. Resuspend the remaining third well of the positive as well as negative control with the antibody mix with the mouse IgG1 isotype control PerCP/Cy5.5. Incubate for 15 min at 4°C.
42. Add 100 µl FACS buffer to each well and mix. Centrifuge the plate for 5 min with 520 × g at 4°C.
43. Remove the supernatant and resuspend the cells in 100 µl FACS buffer. Centrifuge the plate for 5 min with 520 × g at 4°C.
44. Remove the supernatant and resuspend the cells in 80 µl Cytofix/Cytoperm Buffer (Cytofix/Cytoperm<sup>TM</sup> Fixation/Permeabilization Kit, BD Biosciences). Incubate for 20 min at 4°C.
45. Add 100 µl FACS buffer and centrifuge for 5 min with 520 × g at 4°C.
46. Remove the supernatant and resuspend the cells in 1 × Perm/Wash buffer. Incubate for 5 min at RT. Centrifuge for 5 min with 520 × g at RT.
47. Remove the supernatant and resuspend the cells in 1 × Perm/Wash buffer. Incubate for 5 min at RT. Centrifuge for 5 min with 520 × g at RT.
48. During the incubation time, prepare the staining mix for intracellular staining of cytokines. Dilute the antibodies according to Table 60 (CD4<sup>+</sup> T cells) or Table 61 (CD8<sup>+</sup> T cells) in 1 × Perm/Wash buffer (50 µl/sample). Prepare an



**Table 62.** Isotype control staining for MLR with naïve CD4<sup>+</sup> T cells

Fluorophore/Labeling	Isotype	Clone	#Catalog	Company	Dilution
PE/Dazzle594	Rat IgG2a	RTK2758	400558	BioLegend	1:200
A647	Mouse IgG1	MOPC-21	400136	BioLegend	1:125
BV605	Mouse IgG1	MOPC-21	400162	BioLegend	1:160
PE	Rat IgG1	G0114F7	401906	BioLegend	1:400
BV421	Mouse IgG1	MOPC-21	400158	BioLegend	1:100
PE/Cy7	Mouse IgG1	MOPC-21	400126	BioLegend	1:200

**Table 63.** Isotype control staining for MLR with naïve CD8<sup>+</sup> T cells

Fluorophore/Labeling	Isotype	Clone	#Catalog	Company	Dilution
PE/Dazzle594	Rat IgG2a	RTK2758	400558	BioLegend	1:200
A647	Mouse IgG1	MOPC-21	400136	BioLegend	1:100
BV605	Mouse IgG1	MOPC-21	400162	BioLegend	1:160
PE	Mouse IgG1	MOPC-21	400140	BioLegend	1:160
BV421	Mouse IgG1	MOPC-21	400158	BioLegend	1:100
PE/Cy7	Mouse IgG1	MOPC-21	400126	BioLegend	1:200

isotype control mix according to Table 62 (CD4<sup>+</sup> T cells) or Table 63 (CD8<sup>+</sup> T cells) in 1 × Perm/Wash buffer for 4 samples.

49. Resuspend the cells (DC with T cells) as well as one well (stained with master mix in extracellular staining mix) of the positive (with CD3/CD28 Dynabeads) and negative (T cells only) controls in 50 µl staining mix. Resuspend the remaining two wells in 50 µl of the isotype control mix. Incubate for 30 min at RT.
50. Add 100 µl 1 × Perm/Wash buffer to each well. Incubate for 5 min at RT.
51. Centrifuge for 5 min with 520 × g at RT. Remove the supernatant and resuspend in 100 µl Perm/Wash buffer. Incubate for 5 min.
52. Centrifuge for 5 min with 520 × g at RT. Remove the supernatant and resuspend in 100 µl Perm/Wash buffer. Incubate for 5 min.
53. Centrifuge for 5 min with 520 × g at RT. Remove the supernatant and resuspend in 100 µl FACS buffer.
54. Centrifuge for 5 min with 520 × g at RT. Remove the supernatant and resuspend in 100 µl FACS buffer.
55. Centrifuge for 5 min with 520 × g at RT. Remove the supernatant and add 100 µl FACS buffer to each well. Acquire the samples on an LSRFortessa (BD Biosciences; Table 57).

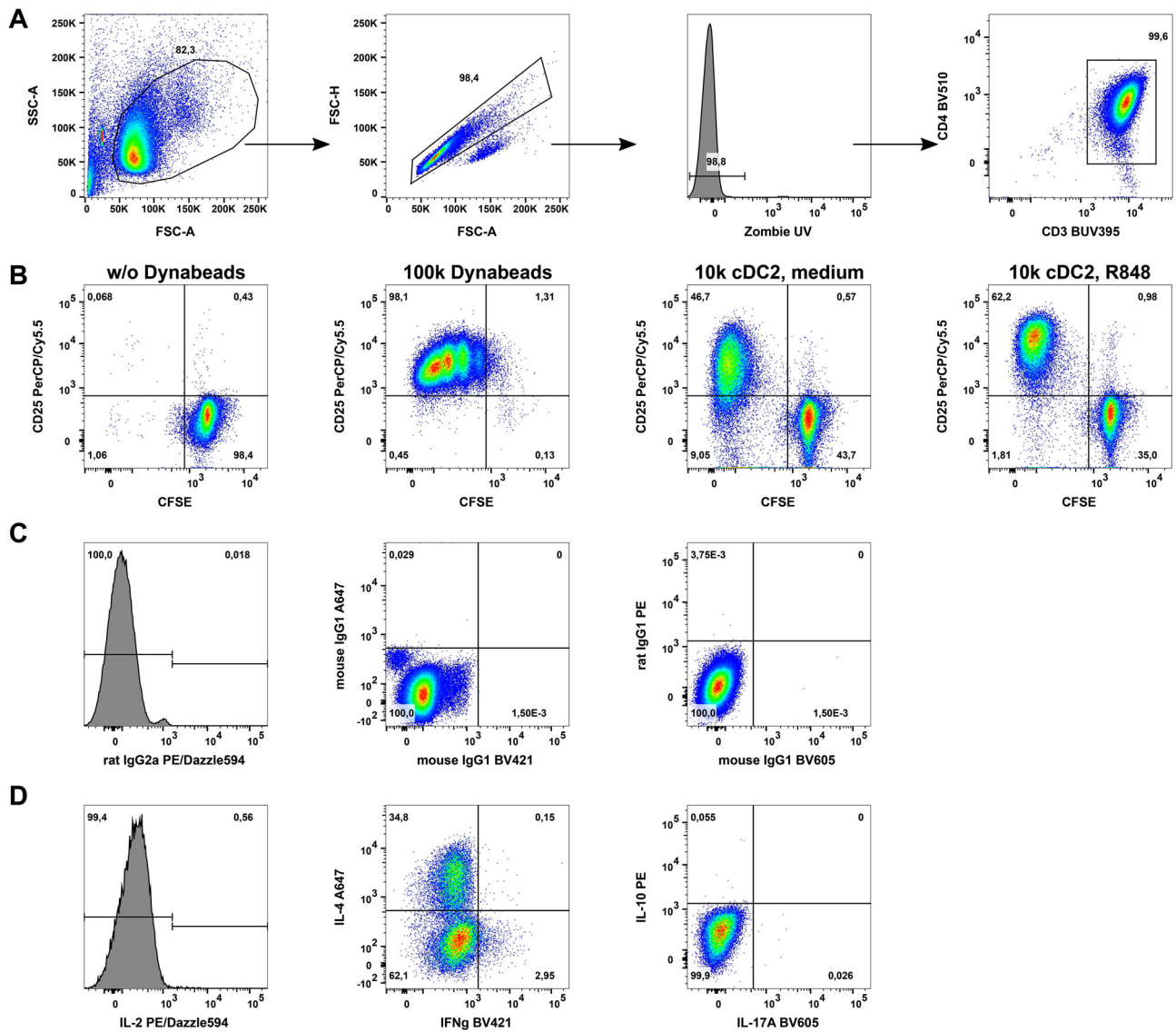
### 13.4 Data analysis

#### 13.4.1 Analysis of naïve CD4<sup>+</sup> T cell proliferation, activation, and polarization

1. Export the files in fcs 3.1 format. Analyze the data using FlowJo (V10) or another software you prefer.
2. For analysis, gate lymphocytes using a morphology gate (FSC-A/SSC-A) as shown in Fig. 31A. Then, select sin-

gle cells using FSC-A/FSC-H. Exclude dead cells using Zombie UV. Gate for CD4<sup>+</sup> T cells using CD3 as well as CD4.

3. To identify proliferated and activated CD4<sup>+</sup> T cells, select the gated CD4<sup>+</sup> T cells and analyze for CFSE as well as CD25. Using a quadrant gate, divide the T cells into not proliferated/not activated (CFSE<sup>+</sup>/CD25<sup>-</sup>), not proliferated/activated (CFSE<sup>+</sup>/CD25<sup>+</sup>), proliferated/not activated (CFSE<sup>-</sup>/CD25<sup>-</sup>) and proliferated/activated (CFSE<sup>-</sup>/CD25<sup>+</sup>) as shown in Fig. 31B. Set the gate using the negative control wells (T cells without Dynabeads without DC). Here, all T cells should be in the CFSE<sup>+</sup>/CD25<sup>-</sup> quadrant (Fig. 31B, left panel).
4. Assign the gating to the positive control well (T cells with 100k Dynabeads) and assure that the majority of the T cells is now in the upper left quadrant (CFSE<sup>-</sup>/CD25<sup>+</sup>) (Fig. 31B, middle panel). If this is the case, transfer the gate to all samples.
5. HLA-mismatched T cells cultured with DC should show proliferation in between negative and positive control. Thus, cells should be in the lower right quadrant corresponding to not proliferated/not activated and cells in the upper left quadrant corresponding to proliferated and activated T cells (see Fig. 31B, right panel). Using the statistics function of FlowJo, export an Excel file with the percentage of proliferated CD4<sup>+</sup> T cells by adding “Frequency of parent” to the CFSE<sup>-</sup> CD25<sup>+</sup> gate.
6. Select the proliferated and activated T cells (CFSE<sup>-</sup>/CD25<sup>+</sup>) and analyze for intracellular cytokines. To account for unspecific binding of the antibodies, use the positive control (T cells with Dynabeads) stained with CD25-PerCP/Cy5.5 in the first staining step and the isotype controls in the second staining step. Based on the fluorescence signal of the isotype controls, set the gates either using the histogram function or using quadrant gates in pseudocolor plots (see Fig. 31C). In the



**Figure 31.** Flow cytometric analysis of CD4<sup>+</sup> T cells after MLR with human cDC2. MLR was performed as described in 13.3.2. After restimulation of T cells, CD4<sup>+</sup> T cells were stained with antibodies (see Table 58). Samples were acquired using an LSRFortessa and analyzed using FlowJo (V10). (A) After a morphology gate (FSC-A/SSC-A), doublets were excluded using (FSC-A/FSC-H). Within living cells (Zombie UV<sup>-</sup>), CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>+</sup>) were gated. (B) CD4<sup>+</sup> T cells gated as in (A) were analyzed for proliferation (CFSE) and activation (CD25). Cells were divided into proliferated and activated (CFSE<sup>-</sup>CD25<sup>+</sup>) T cells and not proliferated, not activated T cells (CFSE<sup>+</sup>CD25<sup>-</sup>) using a quadrant gate. (C-D) Proliferated and activated T cells (CFSE<sup>-</sup>CD25<sup>+</sup>; upper left quadrant in (B)) as gated in B were analyzed for production of cytokines by intracellular flow cytometry. According to the signal of isotype controls (C), gates were set and transferred to (D) samples stained with specific antibodies for the cytokines (see Table 60).

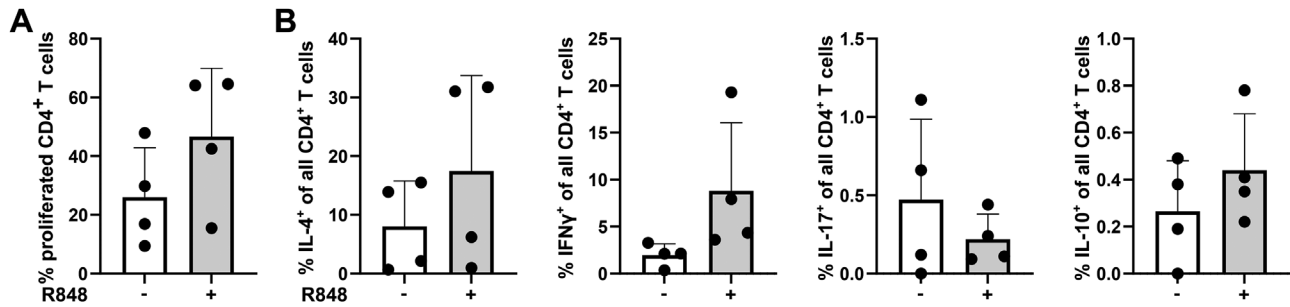
positive gates, less than 1% positive cells should be contained for the isotype controls.

7. Apply the gating for the cytokines to all samples and add “Frequency of grandparents” or “Frequency of parents” to each of the gated cytokine<sup>+</sup> populations. Export the frequency using the Table function of FlowJo.
8. In Excel, correct for the signal of the isotype control by subtracting the percentage of positive cells in the T cell controls stained with isotypes from each of the samples stained with antibodies against the different cytokines.
9. The results of proliferation as well as cytokine production of the T cells can now be plotted using GraphPad Prism or any

other preferred software (see Fig. 32 as an example). Summarized results are shown in Table 64.

### 13.4.2 Analysis of naïve CD8<sup>+</sup> T cell proliferation, activation, and phenotype

10. Export the files in fcs 3.1 format. Analyze the data using FlowJo (V10) or another software you prefer.
11. For analysis, gate lymphocytes using a morphology gate (FSC-A/SSC-A) as shown in Fig. 33A. Then, select single



**Figure 32.** Measuring proliferation and polarization of CD4<sup>+</sup> T cells after MLR with human cDC2. MLR was performed as described in 13.3.2. CD4<sup>+</sup> T cells were analyzed as shown in Fig. 31. For each population frequency of parent (Fig. 31B) or frequency of grandparent (Fig. 31C-D) was determined and exported using FlowJo (V10). (A) Frequency of proliferated CD4<sup>+</sup> T cells was plotted as bar graphs (+ standard deviation (SD); individual donors shown as filled circles) using GraphPad Prism (V9). (B) Frequency of cytokine<sup>+</sup> cells among all CD4<sup>+</sup> T cells was plotted as bar graphs (+ standard deviation (SD); individual donors shown as filled circles) using GraphPad Prism (V9).

cells using FSC-A/FSC-H. Exclude dead cells using Zombie UV. Gate for CD8<sup>+</sup> T cells using CD3 as well as CD8.

- To identify proliferated CD8<sup>+</sup> T cells, select the gated CD8<sup>+</sup> T cells and analyze for CFSE using the histogram function. Divide the T cells into not proliferated (CFSE<sup>+</sup>) and proliferated (CFSE<sup>-</sup>) cells as shown in Fig. 31B. Set the gate using the negative control wells (T cells without Dynabeads without DC). Here, all T cells should be CFSE<sup>+</sup> (Fig. 33B, left panel).
- Assign the gating to the positive control well (T cells with 100k Dynabeads) and assure that the majority of the T cells is now in the CFSE<sup>-</sup> gate (Fig. 33B, middle panel). If this is the case, transfer the gate to all samples.
- HLA-mismatched T cells cultured with DC should show proliferation in between the negative and positive control. Thus, some cells should be in the CFSE<sup>+</sup> gate corresponding to not proliferated cells, while other cells should be negative for CFSE corresponding to proliferated CD8<sup>+</sup> T cells (see Fig. 33B, right panel). Using the statistics function of FlowJo, export an Excel file with the percentage of proliferated CD8<sup>+</sup> T cells by adding “Frequency of parent” to the CFSE<sup>-</sup> gate.
- Select the proliferated CD8<sup>+</sup> T cells (CFSE<sup>-</sup>) and analyze for intracellular cytokines. To account for unspecific binding of the antibodies, use the positive control (T cells with Dynabeads) stained with the isotype controls in the second staining step. Based on the fluorescence signal of the isotype controls, set the gates either using the histogram function or using quadrant gates in pseudocolor plots (see Fig. 33C).

In the positive gates, less than 1% positive cells should be contained for the isotype controls.

- Apply the gating for the cytokines to all samples and add “Frequency of grandparents” or “Frequency of parents” to each of the gated cytokine<sup>+</sup> populations. Export the frequency using the Table function of FlowJo.
- In Excel, correct for the signal of the isotype control by subtracting the percentage of positive cells in the T cell controls stained with isotypes from each of the samples stained with antibodies against the different cytokines.
- The results of proliferation as well as cytokine production of the T cells can now be plotted using GraphPad Prism or any other preferred software (see Fig. 34 as an example). Summarized results are shown in Table 64.

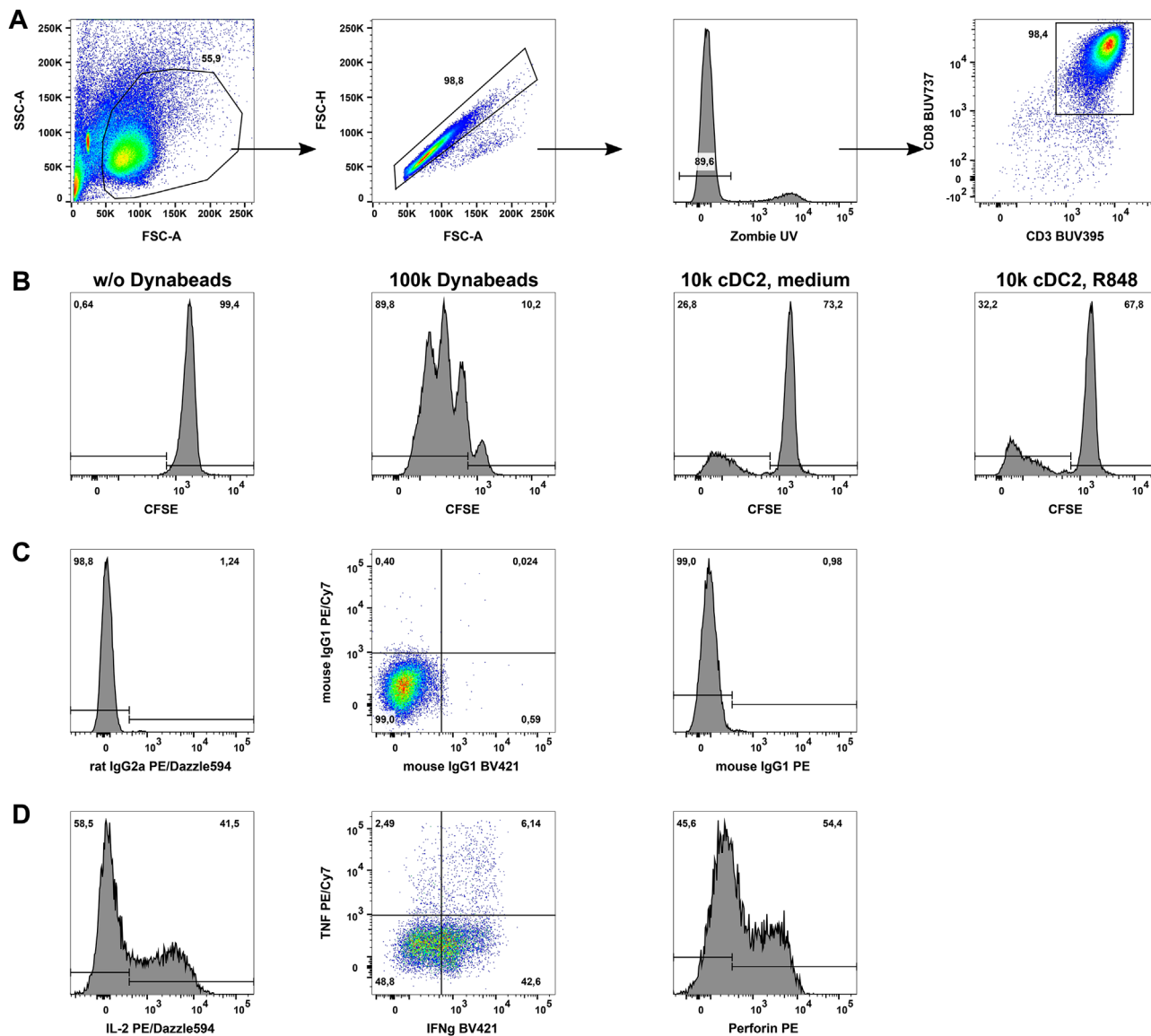
### 13.5 Pitfalls

**Problem: T cells did not proliferate after co-culture with DC**  
**Potential solutions:**

1 of 1,000-10,000 T cells recognizes foreign MHC. Thus, using 100,000 T cells for the assay should induce a clearly measurable T cell response. In case, no proliferation (< 1% proliferated T cells) was observed, positive control wells should be analyzed. If also the T cells cultured with 100,000 Dynabeads did not proliferate, viability or functionality of the T cells was impaired. Thus, T cell isolation should be repeated with fresh buffers, and T cell assay performed with fresh medium. If the T cells cultured with 100,000 Dynabeads proliferated (more than 80% proliferated T cells), then

**Table 64.** Summary of obtained results with naïve CD4<sup>+</sup> (13.4.1) and CD8<sup>+</sup> (13.4.2) T cells

Experimental condition	Read out of the assays	
	Proliferation	Cytokine production
CD4 <sup>+</sup> T cells	TLR-stimulation of cDC2 enhanced T cell proliferation	Increased Th1 and Th2 polarization with TLR-treated cDC2
CD8 <sup>+</sup> T cells	TLR-stimulation of cDC2 enhanced T cell proliferation	Increased production of IFN $\gamma$ , IL-2 as well as Perforin after co-culture with TLR-treated cDC2



**Figure 33.** Flow cytometric analysis of CD8<sup>+</sup> T cells after MLR with human cDC2. MLR was performed as described in 13.3.2. After restimulation of T cells, CD8<sup>+</sup> T cells were stained with antibodies (see Table 59). Samples were acquired using an LSRFortessa and analyzed using FlowJo (V10). (A) After a morphology gate (FSC-A/SSC-A), doublets were excluded using (FSC-A/FSC-H). Within living cells (Zombie UV<sup>-</sup>), CD8<sup>+</sup> T cells (CD3<sup>+</sup>CD8<sup>+</sup>) were gated. (B) CD8<sup>+</sup> T cells gated as in (A) were analyzed for proliferation (CFSE). Cells were divided into proliferated (CFSE<sup>-</sup>) T cells and not proliferated T cells (CFSE<sup>+</sup>) using a threshold according to the negative control (left panel). (C-D) Proliferated T cells (CFSE<sup>-</sup>) as gated in (B) were analyzed for production of cytokines by intracellular flow cytometry. According to the signal of isotype controls (C), gates were set and transferred to (D) samples stained with specific antibodies for the cytokines (see Table 61).

most probably T cells were cultured with autologous instead of allogeneic DC. Repeat the assay and make sure to culture DC with T cells from an HLA-mismatched donor.

#### **Problem: Discrimination of proliferated and not proliferated T cells is not possible**

##### **Potential solutions:**

In case discrimination of proliferated and not proliferated T cells is difficult, staining with CFSE has to be optimized. To allow for discrimination, CFSE-staining of the T cells has to be high enough. Make sure that T cells were washed with PBS prior to

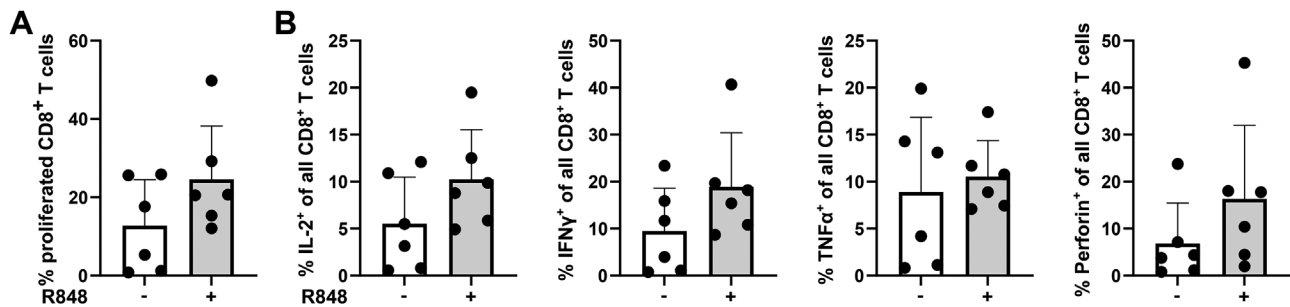
the CFSE-staining to remove proteins, which are present in the buffer. Make sure that the correct number of cells was used during CFSE-staining. If the cell concentration is too high, T cells will only be weakly labeled with CFSE, which impairs the differentiation of proliferated and not proliferated cells.

#### **Problem: High background values for isotype controls**

##### **Potential solutions:**

If high background values for isotype controls were observed, either washing of the samples was not performed accordingly or the concentration of the isotype control was too high. As the





**Figure 34.** Measuring proliferation and cytokine production of CD8<sup>+</sup> T cells after MLR with human cDC2. MLR was performed as described in 13.3.2. CD8<sup>+</sup> T cells were analyzed as shown in Fig. 33. For each population frequency of parent (Fig. 33B) or frequency of grandparent (Fig. 33C-D) was determined and exported using FlowJo (V10). (A) Frequency of proliferated CD8<sup>+</sup> T cells was plotted as bar graphs (+ standard deviation (SD); individual donors shown as filled circles) using GraphPad Prism (V9). (B) Frequency of cytokine<sup>+</sup> cells among all CD8<sup>+</sup> T cells was plotted as bar graphs (+ standard deviation (SD); individual donors shown as filled circles) using GraphPad Prism (V9).

Perm/Wash buffer in the used kit contains saponin, small holes in the membrane are built for the intracellular staining process. To allow unbound antibody to exit the cells, two washing steps should be performed with Perm/Wash buffer and an incubation time of 5 min. Further, make sure that the concentration of the isotype controls matches the concentration of the antibody. As antibodies against intracellular cytokines often have concentrations less than 50  $\mu\text{g/ml}$ , the isotype control either has to have the same concentration or the dilution has to be adapted. E.g. in case the antibody has a concentration of 50  $\mu\text{g/ml}$  and is used in 1:50 dilution, then an isotype control with 200  $\mu\text{g/ml}$  has to be diluted 1:200.

### 13.6 Top tricks

This section describes how to analyze the capacity of human DC to induce naïve T cell responses. As it becomes more and more aware that sustained antigen stimulation can lead to T cell exhaustion [200], marker of T cell exhaustion (e.g. CD223 (LAG-3), CD279 (PD-1), CD366 (Tim-3)) can be added to the extracellular antibody staining mix (Tables 60 and 61). Further, while here an exemplary TLR ligand (R848) was used, which was shown before to strongly activate human primary DC [4, 153, 199, 201], also other TLR ligands as well as tolerance-inducing ligands might be used. In addition, blocking antibodies against select cytokines (e.g. IL-12) might be added during the co-culture of the DC and T cells in order to determine the impact of select cytokines on proliferation and polarization of the T cells.

In this protocol, DC and T cells were co-cultured for six days (13.3.2.4, step 32), which leads to strong proliferation of the responding T cells. In order to obtain more information about the stimulatory capacity of the different DC subpopulations, the co-culture of DC and T cells might be reduced to a shorter period (e.g., 3 days). Thereby, influences of enhanced expression of MHC-I and MHC-II molecules as well as co-stimulatory molecules on cDC compared to pDC might be observed. However, shorter period of co-culture might also affect production of cytokines as well as expression of exhaustion marker on the analysed T cells.

### 13.7 Summary table

Assay read out for co-culture of TLR-treated cDC2 and allogeneic naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells is summarized in Table 64

**Acknowledgments:** Sections 1, 9, 12 and 13: The Dudziak laboratory was supported by the German Research Foundation [Deutsche Forschungsgemeinschaft (DFG)] (CRC1181-TPA7 261193037, DU548/5-1 420943261, TRR305-TPB5 429280966, RTG2504 401821119, RTG2599 421758891), the Interdisziplinäres Zentrum für klinische Forschung (IZKF) (IZKF-A80, IZKF-A87) and the Bavarian State Ministry of Science and Art (Bayresq.Net-IRIS). Diana Dudziak was funded by the Agence Nationale de la Recherche (ANR) and the DFG (DU548/6-1 431402787).

Section 2: Dr. Luciana Berod received intramural funding from ReALity, a joint initiative of all principal investigators of the Faculty of Biology, the Focus Program Translational Neurosciences (FTN), the Research Center for Immunotherapy (FZI), and the Center for Translational Vascular Biology (CTVB) at Johannes Gutenberg University Mainz.

Section 4: This work was supported by the Deutsche Forschungsgemeinschaft (DFG), grant numbers SFB 1292-Q1 and TE599/3-1 to S.T., SFB 1292-TP11 and DI 2471/1-1 to U.D., SFB 1292-TP20N and CL 419/2-2 to B.E.C., BA 5939/2-1 to R.A.B., as well as by the Research Center for Immunotherapy (FZI, *Forschungszentrum für Immuntherapie*) of the Johannes Gutenberg University Mainz.

Section 5: This work was supported by the Deutsche Forschungsgemeinschaft (DFG HI1103/1-1) to T.H.

Section 6: We thank Kasia Stefanowski for technical assistance, and the Core Facility Bioimaging of the Biomedical Center (BMC) of the Ludwig-Maximilian University for support. This work was supported by the Peter Hans Hofschneider Professorship of the Stiftung Experimentelle Biomedizin (to J.R.), by the DFG (Collaborative Research Center SFB914, project A12; to J.R.) and by the

LMU Institutional Strategy LMU-Excellent within the framework of the German Excellence Initiative (to J.R).

Section 7: This work was supported by the Human Frontier Science Program (HFSP) RGP0032-2022, Deutsche Forschungsgemeinschaft (DFG) grant number 335447717-SFB 1328 (project A20), and Forschungszentrum Medizintechnik Hamburg (FMTHH) 04fmthh2021 to P.J.S.

Section 8: We thank members of the Clausen laboratory for fruitful discussions and critical reading of the manuscript. Work in the Clausen laboratory is supported by grants from the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) to B.E.C. (CL 419/2-2 [Project Nr. 315501751], CL 419/4-1, CL 419/7-1 [Project Nr. 503972215], and SFB1292/2 TP20N [Project Nr. 318346496]) and R.A.B. (BA 5939/2-1). B.E.C. and R.A.B. are members of the Research Center for Immunotherapy (Forschungszentrum Immuntherapie, FZI) of the University Medical Center Mainz.

Section 10: This work was supported by the German Research Foundation (DFG): Ma1924/9-2

Section 11: This work was funded by Deutsche Forschungsgemeinschaft under Germany's Excellence Strategy EXC2151 (Project 390873048) to S.B.

Open access funding enabled and organized by Projekt DEAL.

**Conflict of interest:** The authors declare no commercial or financial conflict of interest.

**Author contributions:** Section 1: Christian H. K. Lehmann\*, Lukas Amon\*, Anna Seichter, Gordon F. Heidkamp, Giorgi Tchitashvili, Nounagnon R. Tochoedo, Damir Vurnek, Lukas Heger, and Diana Dudziak. \* Contributed equally; lead author Diana Dudziak

Section 2: Gloria J. Godoy, Lucila Giusiano, Tim Sparwasser and Luciana Berod; lead author Luciana Berod

Section 3: Regine J. Dress, Wan Ting Kong, Charles-Antoine Dutertre and Florent Ginhoux; lead author Florent Ginhoux

Section 4: Anna Gabele, Malte Sielaff, Anna Brand, Tobias Bopp, Björn E. Clausen, Ronald A. Backer, Stefan Tenzer, Ute Distler; lead authors Stefan Tenzer and Ute Distler

Section 5: Ahmed E.I. Hamouda, Melanie Geiger, Thomas Hieronymus; lead author Thomas Hieronymus

Section 6: Madeleine T. Schmitt, Rifat A. Reza, Jörg Renkawitz; lead author Jörg Renkawitz

Section 7: Lola C. Hernandez, Tamara López-López, Pablo J. Sáez; lead author Pablo J. Sáez

Section 8: Christina Eich, Anna Brand, Ronald A. Backer, Patrizia Stoitzner, Björn E. Clausen; lead author Björn E. Clausen

Section 9: Lukas Heger, Lukas Hatscher, Tomasz Kaszubowski, and Diana Dudziak; lead author Diana Dudziak

Section 10: Luxia Chen\*, Meihong Da\*, Karsten Mahnke, \*Equal contribution; lead author Karsten Mahnke

Section 11: Laura Schlautmann, Dominik Nitsche, Fabian Zink and Sven Burgdorf; lead author Sven Burgdorf

Section 12: Christian H. K. Lehmann\*, Lukas Amon\*, Anna Seichter, Giorgi Tchitashvili, Nounagnon R. Tochoedo, Lukas

Jacobi, Lukas Heger, and Diana Dudziak; \* Contributed equally; lead author Diana Dudziak

Section 13: Lukas Heger, Lukas Hatscher, Tomasz Kaszubowski, and Diana Dudziak, lead author Diana Dudziak

**Data availability statement:** Section 4: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the jPOST partner repository<sup>25</sup> and can be accessed using the dataset identifiers PXD031960 (ProteomeXchange) and JPST001508 (jPOST). For all other sections the data that support the findings of this study are available from the corresponding author(s) upon reasonable request.

**Peer review:** The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.202249925>

## References

- Amon, L., Lehmann, C. H. K., Baranska, A., Schoen, J., Heger, L. and Dudziak, D., Transcriptional control of dendritic cell development and functions. *Int Rev Cell Mol Biol.* 2019. **349**: 55–151.
- Dudziak, D., Kamphorst, A. O., Heidkamp, G. F., Buchholz, V. R., Trumppheller, C., Yamazaki, S., Cheong, C. et al., Differential antigen processing by dendritic cell subsets in vivo. *Science.* 2007. **315**: 107–111.
- Lehmann, C. H. K., Baranska, A., Heidkamp, G. F., Heger, L., Neubert, K., Luhr, J. J., Hoffmann, A. et al., DC subset-specific induction of T cell responses upon antigen uptake via Fcγ receptors in vivo. *J. Exp. Med.* 2017. **214**: 1509–1528.
- Heger, L., Balk, S., Luhr, J. J., Heidkamp, G. F., Lehmann, C. H. K., Hatscher, L., Purbojo, A. et al., CLEC10A Is a Specific Marker for Human CD1c(+) Dendritic Cells and Enhances Their Toll-Like Receptor 7/8-Induced Cytokine Secretion. *Front. Immunol.* 2018. **9**: 744.
- Liu, H. and Johnston, A. P., A programmable sensor to probe the internalization of proteins and nanoparticles in live cells. *Angew. Chem. Int. Ed Engl.* 2013; **52**: 5744–5748.
- Sonnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J. and Zerial, M., Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* 2000. **149**: 901–914.
- Mellman, I., Plutner, H. and Ukkonen, P., Internalization and rapid recycling of macrophage Fc receptors tagged with monovalent antireceptor antibody: possible role of a prelysosomal compartment. *J. Cell Biol.* 1984. **98**: 1163–1169.
- Reuter, A., Panozza, S. E., Macri, C., Dumont, C., Li, J., Liu, H., Segura, E. et al., Criteria for dendritic cell receptor selection for efficient antibody-targeted vaccination. *J. Immunol.* 2015. **194**: 2696–2705.
- Amon, L., Dudziak, D., Backer, R. A., Clausen, B. E., Gmeiner, C., Heger, L., Jacobi, L. et al., Guidelines for DC preparation and flow cytometry analysis of mouse lymphohematopoietic tissues. *Eur. J. Immunol.* 2023. **53**: 2249893.
- Minarieta, L., Velasquez, L. N., Sparwasser, T. and Berod, L., Dendritic cell metabolism: moving beyond in vitro-culture-generated paradigms. *Curr. Opin. Biotechnol.* 2021. **68**: 202–212.
- Helft, J., Böttcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B., Goubau, D. et al., GM-CSF Mouse Bone Marrow Cultures Comprise a

- Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity*. 2015. **42**: 1197–1211.
- 12 Krawczyk, C. M., Holowka, T., Sun, J., Blagih, J., Amiel, E., DeBerardinis, R. J., Cross, J. R. et al., Toll-like receptor-induced changes in glycolytic metabolism regulate dendritic cell activation. *Blood*. 2010. **115**: 4742–4749.
  - 13 Naik, S., Proietto, A., Wilson, N., Dakic, A., Schnorrer, P., Fuchsberger, M., Lahoud, M. et al., Cutting edge: generation of splenic CD8+ and CD8-dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. *J. Immunol.* 2005. **174**: 6592–6597.
  - 14 Divakaruni, A. S., Paradise, A., Ferrick, D. A., Murphy, A. N. and Jastroch, M., Analysis and Interpretation of Microplate-Based Oxygen Consumption and pH Data. *Methods Enzymol.* 2014. **547**: 309–354.
  - 15 Nolfi-Donagan, D., Braganza, A. and Shiva, S., Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox. Biol.* 2020. **37**: 101674.
  - 16 Mazzucotelli, C., Goñi, M. G., Roura, S. I., González-Aguilar, G. and Ayala-Zavala, J. F., Nitric oxide. *Postharvest Manag. Approaches Maint. Qual. Fresh Prod.* 2016. **169**: 17–36.
  - 17 Dress, R. J., Liu, Z. and Ginhoux, F., Towards the better understanding of myelopoiesis using single-cell technologies. *Mol. Immunol.* 2020. **122**: 186–192.
  - 18 Ginhoux, F., Yalin, A., Dutertre, C. A. and Amit, I., Single-cell immunology: Past, present, and future. *Immunity*. 2022. **55**: 393–404.
  - 19 Andrews, T. S., Kiselev, V. Y., McCarthy, D. and Hemberg, M., Tutorial: guidelines for the computational analysis of single-cell RNA sequencing data. *Nat. Protoc.* 2021. **16**: 1–9.
  - 20 Hie, B., Peters, J., Nyquist, S. K., Shalek, A. K., Berger, B. and Bryson, B. D., Computational Methods for Single-Cell RNA Sequencing. *Annu Rev Biomed Data Sci* 2020. **3**: 339–364.
  - 21 Luecken, M. D. and Theis, F. J., Current best practices in single-cell RNA-seq analysis: a tutorial. *Mol. Syst. Biol.* 2019. **15**: e8746.
  - 22 Lotfollahi, M., Naghipourfar, M., Luecken, M. D., Khajavi, M., Büttner, M., Wagenstetter, M., Avsec, Ž. et al., Mapping single-cell data to reference atlases by transfer learning. *Nat. Biotechnol.* 2021.
  - 23 Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., Hao, Y. et al., Comprehensive Integration of Single-Cell Data. *Cell*. 2019. **177**.
  - 24 Browaeys, R., Saelens, W. and Saeys, Y., NicheNet: modeling intercellular communication by linking ligands to target genes. *Nat. Methods*. 2020. **17**: 159–162.
  - 25 Wolf, F. A., Angerer, P. and Theis, F. J., SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol.* 2018. **19**: 15.
  - 26 Picelli, S., Faridani, O. R., Björklund, Å. K., Winberg, G., Sagasser, S. and Sandberg, R., Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* 2014. **9**: 171–181.
  - 27 Cossarizza, A., Chang, H., Radbruch, A., Abrignani, S., Addo, R., Akdis, M., Andrä, I. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *Eur. J. Immunol.* 2021. **51**: 2708–3145.
  - 28 Bo, L. and Dewey, C. N., RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011. **12**: 323.
  - 29 Becht, E., McInnes, L., Healy, J., Dutertre, C. A., Kwok, I. W. H., Ng, L. G., Ginhoux, F. et al., Dimensionality reduction for visualizing single-cell data using UMAP. *Nat. Biotechnol.* 2019. **37**: 38–44.
  - 30 Mulder, K., Patel, A. A., Kong, W. T., Piot, C., Halitzki, E., Dunsmore, G., Khalilzad, S. et al., Cross-tissue single-cell landscape of human monocytes and macrophages in health and disease. *Immunity*. 2021. **54**: 1883–1900. e5.
  - 31 Brown, C. C., Gudjonson, H., Pritykin, Y., Deep, D., Lavallée, V. P., Mendoza, A., Fromme, R. et al., Transcriptional Basis of Mouse and Human Dendritic Cell Heterogeneity. *Cell* 2019. **179**: 846–863. e24.
  - 32 Satpathy, A. T., Briseño, C. G., Lee, J. S., Ng, D., Manieri, N. A., Kc, W., Wu, X. et al., Notch2-Dependent Classical Dendritic Cells Orchestrate Intestinal Immunity to Attaching-and-Effacing Bacterial Pathogens. *Nat. Immunol.* 2013. **14**: 937–948.
  - 33 Lewis, K. L., Caton, M. L., Bogunovic, M., Greter, M., Grajkowska, L. T., Ng, D., Klinakis, A. et al., Notch2 Receptor Signaling Controls Functional Differentiation of Dendritic Cells in the Spleen and Intestine. *Immunity* 2011. **35**: 780–791.
  - 34 Gao, Y., Nish, S. A., Jiang, R., Hou, L., Licona-Limón, P., Weinstein, J. S., Zhao, H. et al., Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells. *Immunity* 2013. **39**: 722–732.
  - 35 Drescher, H., Weiskirchen, S. and Weiskirchen, R., Flow Cytometry: A Blessing and a Curse. *Biomedicines* 2021. **9**: 1613.
  - 36 Jaye, D. L., Bray, R. A., Gebel, H. M., Harris, W. A. C. and Waller, E. K., Translational Applications of Flow Cytometry in Clinical Practice. *J. Immunol.* 2012. **188**: 4715–4719.
  - 37 Bludau, I. and Aebersold, R., Proteomic and Interactomic Insights into the Molecular Basis of Cell Functional Diversity. *Nat. Rev. Mol. Cell Biol.* 2020. **21**: 327–340.
  - 38 Aebersold, R. and Mann, M., Mass-Spectrometric Exploration of Proteome Structure and Function. *Nature* 2016. **537**: 347–355.
  - 39 Myers, S. A., Rhoads, A., Cocco, A. R., Peckner, R., Haber, A. L., Schweitzer, L. D., Krug, K. et al., Streamlined Protocol for Deep Proteomic Profiling of FAC-Sorted Cells and Its Application to Freshly Isolated Murine Immune Cells. *Mol. Cell. Proteomics* 2019. **18**: 995–1009.
  - 40 Maes, E., Cools, N., Willems, H. and Baggerman, G., FACS-Based Proteomics Enables Profiling of Proteins in Rare Cell Populations. *Int. J. Mol. Sci.* 2020. **21**: 1–12.
  - 41 Sielaff, M., Kuharev, J., Bohn, T., Hahlbrock, J., Bopp, T., Tenzer, S. and Dittler, U., Evaluation of FASP, SP3, and IST Protocols for Proteomic Sample Preparation in the Low Microgram Range. *J. Proteome Res.* 2017. **16**: 4060–4072.
  - 42 Hughes, C. S., Moggridge, S., Müller, T., Sorensen, P. H., Morin, G. B. and Krijgsvelde, J., Single-Pot, Solid-Phase-Enhanced Sample Preparation for Proteomics Experiments. *Nat. Protoc.* 2019. **14**: 68–85.
  - 43 Hughes, C. S., Foehr, S., Garfield, D. A., Furlong, E. E., Steinmetz, L. M. and Krijgsvelde, J., Ultrasensitive Proteome Analysis Using Paramagnetic Bead Technology. *Mol. Syst. Biol.* 2014. **10**: 757.
  - 44 Meier, F., Brunner, A. D., Frank, M., Ha, A., Bludau, I., Voytk, E., Kaspar-Schoenefeld, S. et al., DiaPASEF: Parallel Accumulation-Serial Fragmentation Combined with Data-Independent Acquisition. *Nat. Methods* 2020. **17**: 1229–1236.
  - 45 Diener, N., Fontaine, J. F., Klein, M., Hieronymus, T., Wanke, F., Kurschus, F. C., Ludwig, A. et al., Posttranslational Modifications by ADAM10 Shape Myeloid Antigen-Presenting Cell Homeostasis in the Splenic Marginal Zone. *Proc. Natl. Acad. Sci. U. S. A.* 2021. **118**: e2111234118.
  - 46 Demichev, V., Messner, C. B., Vernardis, S. I., Lilley, K. S. and Ralser, M., DIA-NN: Neural Networks and Interference Correction Enable Deep Proteome Coverage in High Throughput. *Nat. Methods* 2020. **17**: 41–44.
  - 47 Kolde, R. Pheatmap: Pretty Heatmaps. 2019.
  - 48 Lazar, C. ImputeLCMD: A Collection of Methods for Left-Censored Missing Data Imputation. 2015.



- 49 Stacklies, W., Redestig, H., Scholz, M., Walther, D. and Selbig, J., PcaMethods—a Bioconductor Package Providing PCA Methods for Incomplete Data. *Bioinformatics* 2007. 23: 1164–1167.
- 50 Huang, D. W., Sherman, B. T. and Lempicki, R. A., Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources. *Nat. Protoc.* 2009. 4: 44–57.
- 51 Moggridge, S., Sorensen, P. H., Morin, G. B. and Hughes, C. S., Extending the Compatibility of the SP3 Paramagnetic Bead Processing Approach for Proteomics. *J. Proteome Res.* 2018. 17: 1730–1740.
- 52 Sinitcyn, P., Hamzeiy, H., Salinas Soto, F., Itzhak, D., McCarthy, F., Wichmann, C., Steger, M. et al., MaxDIA Enables Library-Based and Library-Free Data-Independent Acquisition Proteomics. *Nat. Biotechnol.* 2021. 39: 1563–1573.
- 53 Navarro, P., Kuharev, J., Gillet, L. C., Bernhardt, O. M., MacLean, B., Röst, H. L., Tate, S. A. et al., A Multicenter Study Benchmarks Software Tools for Label-Free Proteome Quantification. *Nat. Biotechnol.* 2016. 34: 1130–1136.
- 54 Distler, U., Kuharev, J., Navarro, P., Levin, Y., Schild, H. and Tenzer, S., Drift Time-Specific Collision Energies Enable Deep-Coverage Data-Independent Acquisition Proteomics. *Nat. Methods* 2014. 11: 167–170.
- 55 Okuda, S., Watanabe, Y., Moriya, Y., Kawano, S., Yamamoto, T., Matsumoto, M., Takami, T. et al., JPOSTrepo: An International Standard Data Repository for Proteomes. *Nucleic. Acids. Res.* 2017. 45: D1107–D1111.
- 56 Murphy, D. A. and Courtneidge, S. A., The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. *Nat. Rev. Mol. Cell Biol.* 2011. 12: 413–426.
- 57 David-Pfeuty, T. and Singer, S. J., Altered distributions of the cytoskeletal proteins vinculin and alpha-actinin in cultured fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA* 1980. 77: 6687–6691.
- 58 Tarone, G., Cirillo, D., Giancotti, F. G., Comoglio, P. M. and Marchisio, P. C., Rous sarcoma virus-transformed fibroblasts adhere primarily at discrete protrusions of the ventral membrane called podosomes. *Exp. Cell. Res.* 1985. 159: 141–157.
- 59 Chen, W. T., Chen, J. M., Parsons, S. J. and Parsons, J. T., Local degradation of fibronectin at sites of expression of the transforming gene product pp60src. *Nature* 1985. 316: 156–158.
- 60 Linder, S. and Wiesner, C., Tools of the trade: podosomes as multipurpose organelles of monocytic cells. *Cell Mol Life Sci.* 2015. 72: 121–135.
- 61 van den Dries, K., Linder, S., Maridonneau-Parini, I. and Poincloux, R., Probing the mechanical landscape - new insights into podosome architecture and mechanics. *J. Cell Sci.* 2019. 132: jcs236828.
- 62 Diaz, B., Invadopodia Detection and Gelatin Degradation Assay. *Bio Protoc.* 2013. 3: e997.
- 63 West, M. A., Wallin, R. P., Matthews, S. P., Svensson, H. G., Zaru, R., Ljunggren, H. G., Prescott, A. R. et al., Enhanced dendritic cell antigen capture via toll-like receptor-induced actin remodeling. *Science* 2004. 305: 1153–1157.
- 64 Burns, S., Hardy, S. J., Buddle, J., Yong, K. L., Jones, G. E. and Thrasher, A. J., Maturation of DC is associated with changes in motile characteristics and adherence. *Cell Motil. Cytoskeleton* 2004. 57: 118–132.
- 65 Worbs, T., Hammerschmidt, S. I. and Förster, R., Dendritic cell migration in health and disease. *Nat. Rev. Immunol.* 2017. 17: 30–48.
- 66 Moreau, H. D., Piel, M., Voituriez, R. and Lennon-Duménil, A. M., Integrating Physical and Molecular Insights on Immune Cell Migration. *Trends Immunol.* 2018. 39: 632–643.
- 67 Nourshargh, S., Hordijk, P. L. and Sixt, M., Breaching multiple barriers: leukocyte motility through venular walls and the interstitium. *Nat. Rev. Mol. Cell Biol.* 2010. 11: 366–378.
- 68 Kameritsch, P. and Renkawitz, J., Principles of Leukocyte Migration Strategies. *Trends Cell Biol.* 2020. 30: 818–832.
- 69 Sixt, M. and Lämmermann, T., In vitro analysis of chemotactic leukocyte migration in 3D environments. *Methods in molecular biology (Clifton, N.J.)*. 2011. 769: 149–165.
- 70 Vargas, P., Chabaud, M., Thiam, H. R., Lankar, D., Piel, M. and Lennon-Duménil, A. M., Study of dendritic cell migration using micro-fabrication. *J. Immunol. Methods.* 2016. 432: 30–34.
- 71 Kroll, J., Ruiz-Fernandez, M. J. A., Braun, M. B., Merrin, J. and Renkawitz, J., Quantifying the Probing and Selection of Microenvironmental Pores by Motile Immune Cells. *Curr Protoc* 2022. 2: e407.
- 72 Renkawitz, J., Reversat, A., Leithner, A., Merrin, J. and Sixt, M., Micro-engineered “pillar forests” to study cell migration in complex but controlled 3D environments. *Methods Cell Biol.* 2018. 147: 79–91.
- 73 Nelson, R. D., Quie, P. G. and Simmons, R. L., Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J. Immunol.* 1975. 115: 1650–1656.
- 74 Heit, B., and Kubes, P., Measuring Chemotaxis and Chemokinesis: The Under-Agarose Cell Migration Assay. *Sci. STKE.* 2003. 2003: pl5–pl5.
- 75 Renkawitz, J. and Sixt, M., Mechanisms of force generation and force transmission during interstitial leukocyte migration. *EMBO Rep.* 2010. 11: 744–750.
- 76 Lämmermann, T., Bader, B. L., Monkley, S. J., Worbs, T., Wedlich-Söldner, R., Hirsch, K., Keller, M. et al., Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature.* 2008. 453: 51–55.
- 77 Renkawitz, J., Schumann, K., Weber, M., Lämmermann, T., Pflücke, H., Piel, M., Polleux, J. et al., Adaptive force transmission in amoeboid cell migration. *Nat. Cell Biol.* 2009. 11: 1438–1443.
- 78 Maiuri, P., Rupprecht, J. F., Wieser, S., Rupprecht, V., Bénichou, O., Carpi, N., Coppey, M. et al., Actin flows mediate a universal coupling between cell speed and cell persistence. *Cell.* 2015. 161: 374–386.
- 79 Malawista, S. E. and Chevance, A. D. B., Random locomotion and chemotaxis of human blood polymorphonuclear leukocytes (PMN) in the presence of EDTA: PMN in close quarters require neither leukocyte integrins nor external divalent cations. *Proc. Nat. Acad. Sci. U.S.A.* 1997. 94: 11577–11582.
- 80 Liu, Y. J., Le Berre, M., Lautenschlaeger, F., Maiuri, P., Callan-Jones, A., Heuzé, M., Takaki, T. et al., Confinement and Low Adhesion Induce Fast Amoeboid Migration of Slow Mesenchymal Cells. *Cell.* 2015. 160: 659–672.
- 81 Lämmermann, T., Renkawitz, J., Wu, X., Hirsch, K., Brakebusch, C. and Sixt, M., Cdc42-dependent leading edge coordination is essential for interstitial dendritic cell migration. *Am. J. Blood Res.* 2009. 113: 5703–5710.
- 82 Weier, A. K., Homrich, M., Ebbinghaus, S., Juda, P., Miková, E., Hauschild, R., Zhang, L. et al., Multiple centrosomes enhance migration and immune cell effector functions of mature dendritic cells. *J. Cell Biol.* 2022. 221: e202107134.
- 83 Bell, G. R. R., Natwick, D. E. and Collins, S. R., Rho GTPases, Methods and Protocols. *Methods Mol Biology.* 2018. 1821: 71–85.
- 84 Kopf, A., Renkawitz, J., Hauschild, R., Girkontaite, I., Tedford, K., Merrin, J., Thorn-Seshold, O. et al., Microtubules control cellular shape and coherence in amoeboid migrating cells. *J. Cell Biol.* 2020. 219: e201907154.
- 85 Gaertner, F., Reis-Rodrigues, P., de Vries, I., Hons, M., Aguilera, J., Riedl, M., Leithner, A. et al., WASp triggers mechanosensitive actin patches to facilitate immune cell migration in dense tissues. *Dev. Cell.* 2021. 57: 47–62.



- 86 Collins, S. R., Yang, H. W., Bongler, K. M., Guignet, E. G., Wandless, T. J. and Meyer, T., Using light to shape chemical gradients for parallel and automated analysis of chemotaxis. *Mol. Syst. Biol.* 2015. **11**: 804–804.
- 87 Vargas, P., Maiuri, P., Bretou, M., Sáez, P. J., Pierobon, P., Maurin, M., Chabaud, M. et al., Innate control of actin nucleation determines two distinct migration behaviours in dendritic cells. *Nat. Cell Biol.* 2016. **18**: 43–53.
- 88 Schumann, K., Lammermann, T., Bruckner, M., Legler, D. F., Polleux, J., Spatz, J. P., Schuler, G. et al., Immobilized chemokine fields and soluble chemokine gradients cooperatively shape migration patterns of dendritic cells. *Immunity* 2010. **32**: 703–713.
- 89 Schwarz, J., Bierbaum, V., Vaahtomeri, K., Hauschild, R., Brown, M., de Vries, I., Leithner, A. et al., Dendritic Cells Interpret Haptotactic Chemokine Gradients in a Manner Governed by Signal-to-Noise Ratio and Dependent on GRK6. *Curr. Biol.* 2017. **27**: 1314–1325.
- 90 Reversat, A., Gaertner, F., Merrin, J., Stopp, J., Tasciyan, S., Aguilera, J., de Vries, I. et al., Cellular locomotion using environmental topography. *Nature*. 2020. **582**: 582–585.
- 91 Kwon, K. W., Park, H., Song, K. H., Choi, J. C., Ahn, H., Park, M. J., Suh, K. Y. et al., Nanotopography-Guided Migration of T Cells. *J. Immunol.* 2012. **189**: 2266–2273.
- 92 Kienle, K., Glaser, K. M., Eickhoff, S., Mihlan, M., Knöpper, K., Reátegui, E., Epple, M. W. et al., Neutrophils self-limit swarming to contain bacterial growth in vivo. *Science*. 2021. **372**: eabe7729.
- 93 Tsai, T. Y. C., Collins, S. R., Chan, C. K., Hadjithodorou, A., Lam, P. Y., Lou, S. S., Yang, H. W. et al., Efficient Front-Rear Coupling in Neutrophil Chemotaxis by Dynamic Myosin II Localization. *Dev. Cell* 2019. **49**: 189–205. e6.
- 94 Yang, H. W., Collins, S. R. and Meyer, T., Locally excitable Cdc42 signals steer cells during chemotaxis. *Nat. Cell Biol.* 2016. **18**: 191–201.
- 95 Hons, M., Kopf, A., Hauschild, R., Leithner, A., Gaertner, F., Abe, J., Renkawitz, J. et al., Chemokines and integrins independently tune actin flow and substrate friction during intranodal migration of T cells. *Nat. Immunol.* 2018. **19**: 606–616.
- 96 Moalli, F., Ficht, X., Germann, P., Vladymyrov, M., Stolp, B., de, V. I., Lyck, R. et al., The Rho regulator Myosin IXb enables nonlymphoid tissue seeding of protective CD8+ T cells. *J. Exp. Med.* 2018. **215**: 1869–1890.
- 97 Rupnick, M. A., Stokes, C. L., Williams, S. K. and Lauffenburger, D. A., Quantitative analysis of random motility of human microvessel endothelial cells using a linear under-agarose assay. *Laboratory Investigation J Technical Methods Pathology*. 1988. **59**: 363–372.
- 98 Bergert, M., Erzberger, A., Desai, R. A., Aspalter, I. M., Oates, A. C., Charras, G., Salbreux, G. et al., Force transmission during adhesion-independent migration. *Nat. Cell Biol.* 2015. **17**: 524–529.
- 99 Logue, J. S., Cartagena-Rivera, A. X., Baird, M. A., Davidson, M. W., Chadwick, R. S. and Waterman, C M., Erk regulation of actin capping and bundling by Eps8 promotes cortex tension and leader bleb-based migration. *Elife* 2015. **4**: e08314.
- 100 Ruprecht, V., Wieser, S., Callan-Jones, A., Smutny, M., Morita, H., Sako, K., Barone, V. et al., Cortical Contractility Triggers a Stochastic Switch to Fast Amoeboid Cell Motility. *Cell*. 2015. **160**: 673–685.
- 101 Singh, S. P., Thomason, P. A., Lilla, S., Schaks, M., Tang, Q., Goode, B. L., Machesky, L. M. et al., Cell-substrate adhesion drives Scar/WAVE activation and phosphorylation by a Ste20-family kinase, which controls pseudopod lifetime. *PLoS Biol.* 2020. **18**: e3000774.
- 102 Busch, B., Weimer, R., Woischke, C., Fischer, W. and Haas, R. Helicobacter pylori interferes with leukocyte migration via the outer membrane protein HopQ and via CagA translocation. *Int. J. Med. Microbiol.* 2015. **305**: 355–364.
- 103 Salzer, E., Cagdas, D., Hons, M., Mace, E. M., Garncarz, W., Petronczki, Ö. Y., Platzer, R. et al., RASGRP1 deficiency causes immunodeficiency with impaired cytoskeletal dynamics. *Nat. Immunol.* 2016. **17**: 1352–1360.
- 104 Schneider, C. A., Rasband, W. S. and Eliceiri, K W., NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods*. 2012. **9**: 671–675.
- 105 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S. et al., Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 2012. **9**: 676–682.
- 106 Zantl, R. and Horn, E., Cell Migration, Developmental Methods and Protocols. *Methods Mol Biology*. 2011. **769**: 191–203
- 107 Tinevez, J. Y., Perry, N., Schindelin, J., Hoopes, G. M., Reynolds, G. D., Laplantine, E., Bednarek, S. Y. et al., TrackMate: An open and extensible platform for single-particle tracking. *Methods*. 2017. **115**: 80–90
- 108 Berg, S., Kutra, D., Kroeger, T., Straehle, C. N., Kausler, B. X., Haubold, C., Schiegg, M. et al., ilastik: interactive machine learning for (bio)image analysis. *Nature methods*. 2019. **16**: 1226–1232.
- 109 Feng, M., Zhou, S., Yu, Y., Su, Q., Li, X. and Lin, W., Regulation of the Migration of Distinct Dendritic Cell Subsets. *Front. Cell Dev. Biol.* 2021. **9**: 635221.
- 110 Sáez, P. J., Sáez, J. C., Lennon-Duménil, A. M. and Vargas, P., Role of calcium permeable channels in dendritic cell migration. *Curr. Opin. Immunol.* 2018. **52**: 74–80.
- 111 Sáez, P. J., Vargas, P., Shoji, K. F., Harcha, P. A., Lennon-Duménil, A. M. and Sáez, J. C., ATP promotes the fast migration of dendritic cells through the activity of pannexin 1 channels and P2×7 receptors. *Sci. Signal* 2017. **10**: eaah7107.
- 112 Barbier, L., Sáez, P. J., Attia, R., Lennon-Dumenil, A. M., Lavi, I., Piel, M. and Vargas, P., Myosin II Activity Is Selectively Needed for Migration in Highly Confined Microenvironments in Mature Dendritic Cells. *Front. Immunol.* 2019. **10**: 747.
- 113 Bretou, M., Sáez, P. J., Sanseau, D., Maurin, M., Lankar, D., Chabaud, M., Spanpanato, C. et al., Lysosome signaling controls the migration of dendritic cells. *Sci Immunol* 2017. **2**: eaak9573.
- 114 Vargas, P., Barbier, L., Sáez, P. J. and Piel, M., Mechanisms for fast cell migration in complex environments. *Curr. Opin. Cell Biol.* 2017. **48**: 72–78.
- 115 Harcha, P. A., Lopez-Lopez, T., Palacios, A. G. and Sáez, P. J., Pannexin Channel Regulation of Cell Migration: Focus on Immune Cells. *Front. Immunol.* 2021. **12**: 750480.
- 116 Hong, W., Yang, B., He, Q., Wang, J. and Weng, Q., New Insights of CCR7 Signaling in Dendritic Cell Migration and Inflammatory Diseases. *Front. Pharmacol.* 2022. **13**: 841687.
- 117 de Winde, C. M., Munday, C. and Acton, S. E., Molecular mechanisms of dendritic cell migration in immunity and cancer. *Med Microbiol Immunol* 2020. **209**: 515–529.
- 118 Weber, M., Hauschild, R., Schwarz, J., Moussion, C., de Vries, I., Legler, D. F., Luther, S. A. et al., Interstitial dendritic cell guidance by haptotactic chemokine gradients. *Science* 2013. **339**: 328–332.
- 119 Bosnjak, B., Do, K. T. H., Forster, R. and Hammerschmidt, S. I., Imaging dendritic cell functions. *Immunol. Rev.* 2022. **306**: 137–163.
- 120 Faure-Andre, G., Vargas, P., Yuseff, M. I., Heuze, M., Diaz, J., Lankar, D., Steri, V. et al., Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. *Science* 2008. **322**: 1705–1710.
- 121 Sáez, P. J., Barbier, L., Attia, R., Thiam, H. R., Piel, M. and Vargas, P., Leukocyte Migration and Deformation in Collagen Gels and Microfabricated Constrictions. *Methods Mol. Biol.* 2018. **1749**: 361–373.

- 122 Ershov, D., Phan, M. S., Pylvanainen, J. W., Rigaud, S. U., Le Blanc, L., Charles-Orszag, A., Conway, J. R. W. et al., TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nat. Methods* 2022. 19: 829–883.
- 123 Romani, N., Clausen, B. E. and Stoitzner, P., Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. *Immunol. Rev.* 2010. 234: 120–141
- 124 Clausen, B. E. and Stoitzner, P., Functional specialization of skin dendritic cell subsets in regulating T cell responses. *Front. Immunol.* 2015. 6: 534.
- 125 Bancherau, J. and Steinmann, R. M., Dendritic cells and the control of immunity. *Nature.* 1998. 392: 245–252
- 126 Kaplan, D. H., Ontogeny and function of murine epidermal Langerhans cells. *Nat. Immunol.* 2017. 18: 1068–1075
- 127 Brand, A., Diener, N., Zahner, S. P., Tripp, C., Backer, R. A., Karram, K., Jiang, A. et al., E-cadherin is Dispensable to Maintain Langerhans Cells in the Epidermis. *J. Invest. Dermatol.* 2020. 140: 132–142
- 128 Kashem, S. W., Igyarto, B. Z., Gerami-Nejad, M., Kumamoto, Y., Mohammed, J. A., Jarrett, E., Drummond, R. A. et al., *Candida albicans* morphology and dendritic cell subsets determine T helper cell differentiation. *Immunity.* 2015. 42: 356–366
- 129 Kautz-Neu, K., Noordegraaf, M., Dinges, S., Bennett, C. L., John, D., Clausen, B. E. and von Stebut, E., Langerhans Cells are negative regulators of the anti-Leishmania response. *J. Exp. Med.* 2011. 208:885–891
- 130 Henri, S., Poulin, L. F., Tamoutounour, S., Ardouin, L., Williams, M., de Bovis, B., Devilard, E. et al., CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J. Exp. Med.* 2010. 207: 189–206
- 131 Williams, M., Dutertre, C. A., Scott, C. L., Mc Govern, N., Sichien, D., Charakarov, S., Van Gassen, S. et al., Unsupervised High-Dimensional Analysis Aligns Dendritic Cells across Tissues and Species. *Immunity.* 2016. 45: 669–684
- 132 Seneschal, J., Jiang, X. and Kupper, T. S., Langerin+ Dermal DC, but not Langerhans cells, are required for effective CD8 mediated immune responses after skin scarification with Vaccina Virus (VACV). *J. Invest. Dermatol.* 2014. 134: 686–694
- 133 Mollah, S. A., Dobrin, J. S., Feder, R. E., Tse, S. W., Matos, I. G., Cheong, C., Steinman, R. M. et al., Flt3L dependence helps define an uncharacterized subset of murine cutaneous dendritic cells. *J. Invest. Dermatol.* 2014. 134: 1265–1275
- 134 Stoitzner, P., Tripp, C. H., Eberhart, A., Price, K. M., Jung, J. Y., Bursch, L., Ronchese, F. et al., Langerhans cells cross-present antigen derived from skin. *Proc. Natl Acad. Sci.* 2006. 103: 7783–7788
- 135 Hain, T., Melchior, F., Kamenjarin, N., Muth, S., Weslati, H., Clausen, B. E., Mahnke, K. et al., Dermal CD207-Negative Migratory Dendritic Cells Are Fully Competent to Prime Protective, Skin Homing Cytotoxic T-Lymphocyte Responses. *J. Invest. Dermatol.* 2019. 139: 422–429
- 136 Miller, H. L., Andhey, P. S., Swiecki, M. K., Rosa, B. A., Zaitsev, K., Villani, A. C., Mitreva, M. et al., Altered ratio of dendritic cell subsets in skin-draining lymph nodes promotes Th2-driven contact hypersensitivity. *Proc. Natl Acad. Sci.* 2021. 118: e2032364118
- 137 Probst, H. C., Stoitzner, P., Amon, L., Backer, R. A., Brand, A., Chen, J., Clausen, B. E. et al., Guidelines for DC preparation and flow cytometry analysis of mouse non-lymphoid tissues. *Eur. J. Immunol.* 2023. 53: 2249819.
- 138 Bell, D. B., Kitajima, M. K., Larson, R. P., Stoklasek, T. A., Dang, K., Sakamoto, K., Wagner, K. U. et al., The transcription factor STAT5 is critical in dendritic cells for the development of TH2 but not TH1 responses. *Nat. Immunol.* 2013. 14: 364–371.
- 139 Kissenpfennig, A., Henri, S., Dubois, B., Laplace-Builhé, C., Perrin, P., Romani, N., Tripp, C. H. et al., Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity.* 2005. 22: 643–654
- 140 Ouchi, T., Nakato, G. and Udey, M. C., EpCAM Expressed by Murine Epidermal Langerhans Cells Modulates Immunization to an Epicutaneously-applied Protein Antigen. *J. Invest. Dermatol.* 2016. 136: 1627–1635.
- 141 Stoitzner, P., Pfaller, K., Stössel, H. and Romani, N., A close-up view of migrating Langerhans cells in the skin. *J. Invest. Dermatol.* 2002. 118: 117–125
- 142 Hatscher, L., Amon, L., Heger, L. and Dudziak, D., Inflammasomes in Dendritic Cells: Friend or Foe? *Immunol. Lett.* 2021. 234: 16–32.
- 143 Broz, P. and Dixit, V. M., Inflammasomes: Mechanism of assembly, regulation and signalling. *Nat. Rev. Immunol.* 2016. 16: 407–420.
- 144 Broz, P., Pelegrin, P. and Shao, F., The gasdermins, a protein family executing cell death and inflammation. *Nat. Rev. Immunol.* 2020. 20: 143–157.
- 145 Shi, J., Zhao, Y., Wang, K., Shi, X., Wang, Y., Huang, H., Zhuang, Y. et al., Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature.* 2015. 526: 660–665.
- 146 Liu, X., Zhang, Z., Ruan, J., Pan, Y., Magupalli, V. G., Wu, H. and Lieberman, J., Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature.* 2016. 535: 153–158.
- 147 Swanson, K. V., Deng, M. and Ting, J. P. Y., The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nat. Rev. Immunol.* 2019. 19: 477–489.
- 148 Chung, Y., Chang, S. H., Martinez, G. J., Yang, X. O., Nurieva, R., Kang, H. S., Ma, L. et al., Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling. *Immunity.* 2009. 30: 576–587.
- 149 Dinarello, C. A., IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J. Allergy Clin. Immunol.* 1999. 103: 11–24.
- 150 Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K. et al., Cloning of a new cytokine that induces. *Nature.* 1995. 378: 88–91
- 151 Latz, E., Xiao, T. S. and Stutz, A., Activation and regulation of the inflammasomes. *Nat. Rev. Immunol.* 2013. 13: 397–411.
- 152 Mangan, M. S. J., Olhava, E. J., Roush, W. R., Seidel, H. M., Glick, G. D. and Latz, E., Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov* 2018. 17: 588–606.
- 153 Hatscher, L., Lehmann, C. H. K., Purbojo, A., Onderka, C., Liang, C., Hartmann, A., Cesnjevar, R. et al., Select hyperactivating NLRP3 ligands enhance the TH1- and TH17-inducing potential of human type 2 conventional dendritic cells. *Sci. Signal* 2021. 14: eabe1757.
- 154 McDaniel, M. M., Kottyan, L. C., Singh, H. and Pasare, C., Suppression of Inflammasome Activation by IRF8 and IRF4 in cDCs Is Critical for T Cell Priming. *Cell Rep.* 2020. 31: 107604.
- 155 Zhivaki, D., Borriello, F., Chow, O. A., Sokol, C. L., Zaroni, I., Kagan Corrospendence, J. C., Doran, B. et al., Inflammasomes within Hyperactive Murine Dendritic Cells Stimulate Long-Lived T Cell-Mediated Antitumor Immunity. *Cell Rep.* 2020. 33: 108381.
- 156 Heger, L., Dudziak, D., Amon, L., Hatscher, L., Kaszubowski, T. and Lehmann, C. H. K., Guidelines for DC preparation and flow cytometric analysis of human lymphohematopoietic tissues. *Eur. J. Immunol.* 2023. 53: 2249917.
- 157 Rodrigues, T. S., de Sá, K. S. G., Ishimoto, A. Y., Becerra, A., Oliveira, S., Almeida, L., Gonçalves, A. V. et al., Inflammasomes are activated in response to SARS-cov-2 infection and are associated with COVID-19 severity in patients. *J. Exp. Med.* 2020. 218: e20201707.

- 158 Storek, K. M. and Monack, D. M., Bacterial recognition pathways that lead to inflammasome activation, *Immunol. Rev.* 2015. **265**: 112–129.
- 159 Chakrabarti, A., Banerjee, S., Franchi, L., Loo, Y. M., Gale, M., Núñez, G. and Silverman, R. H., RNase L activates the NLRP3 inflammasome during viral infections, *Cell Host Microbe.* 2015. **17**: 466–477.
- 160 Wang, X., Jiang, W., Yan, Y., Gong, T., Han, J., Tian, Z. and Zhou, R., RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway, *Nat. Immunol.* 2014. **15**: 1126–1133.
- 161 Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S. and Beyaert, R., Stimulation of Toll-like receptor 3 and 4 induces interleukin-1 $\beta$  maturation by caspase-8, *J. Exp. Med.* 2008. **205**: 1967–1973.
- 162 Gaidt, M. M., Ebert, T. S., Chauhan, D., Schmidt, T., Schmid-Burgk, J. L., Rapino, F., Robertson, A. A. B. et al., Human Monocytes Engage an Alternative Inflammasome Pathway, *Immunity.* 2016. **44**: 833–846.
- 163 Shi, J., Zhao, Y., Wang, Y., Gao, W., Ding, J., Li, P., Hu, L. et al., Inflammatory caspases are innate immune receptors for intracellular LPS, *Nature.* 2014. **514**: 187–192.
- 164 Zanoni, I., Tan, Y., Di Gioia, M., Broggi, A., Ruan, J., Shi, J., Donado, C. A. et al., An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells. *Science (80-.)*. 2016. **352**: 1232–1236.
- 165 Heger, L., Amon, L., Lehmann, C. H. K. and Dudziak, D., Systems Immunology Approaches for Understanding of Primary Dendritic Cell Subpopulations in the Past, Present and Future. in: *Syst. Med.*, Elsevier, 2021: 501–510.
- 166 Baker, P. J., Boucher, D., Bierschenk, D., Tebartz, C., Whitney, P. G., D'Silva, D. B., Tanzer, M. C. et al., NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5, *Eur. J. Immunol.* 2015. **45**: 2918–2926.
- 167 Schmid-Burgk, J. L., Gaidt, M. M., Schmidt, T., Ebert, T. S., Bartok, E. and Hornung, V., Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells, *Eur. J. Immunol.* 2015. **45**: 2911–2917.
- 168 Mahnke, K., Guo, M., Lee, S., Sepulveda, H., Swain, S. L., Nussenzweig, M. and Steinman, R. M., The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. *J. Cell Biol.* 2000. **151**: 673–684.
- 169 Inaba, K., Turley, S., Yamaide, F., Iyoda, T., Mahnke, K., Inaba, M., Pack, M. et al., Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. *J. Exp. Med.* 1998. **188**: 2163–2173.
- 170 Mellman, I., Antigen processing and presentation by dendritic cells: cell biological mechanisms. *Adv. Exp. Med. Biol.* 2005. **560**: 63–67.
- 171 Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Untermaehr, J., Steinman, R. M. and Mellman, I., Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* 2000. **288**: 522–527.
- 172 Mahnke, K., Qian, Y., Fondel, S., Brueck, J., Becker, C. and Enk, A. H., Targeting of antigens to activated dendritic cells in vivo cures metastatic melanoma in mice. *Cancer Res.* 2005. **65**: 7007–7012.
- 173 Berard, M. and Tough, D. F., Qualitative differences between naive and memory T cells. *Immunology* 2002. **106**: 127–138.
- 174 Spörri, R. and Reis e Sousa, C., Newly activated T cells promote maturation of bystander dendritic cells but not IL-12 production. *J. Immunol.* 2003. **171**: 6406–6413.
- 175 Szajnik, M., Szczepanski, M. J., Czystowska, M., Elishaev, E., Mandapathil, M., Nowak-Markwitz, E., Spaczynski, M. et al., TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. *Oncogene* 2009. **28**: 4353–4363.
- 176 McCall, J. L., Varney, M. E., Rice, E., Dziadowicz, S. A., Hall, C., Blethen, K. E., Hu, G. et al., Prenatal cadmium exposure alters proliferation in mouse CD4<sup>+</sup> T cells via LncRNA Snhg7. *Front. Immunol.* 2021. **12**: 720635.
- 177 Denham, E. M., Barton, M. I., Black, S. M., Bridge, M. J., de Wet, B., Paterson, R. L., van der Merwe, P. A. et al., A generic cell surface ligand system for studying cell–cell recognition. *PLoS Biol.* 2019. **17**: e3000549.
- 178 Day, C. L., Abrahams, D. A., Lerumo, L., van Rensburg, E. J., Stone, L., O'rie, T., Pienaar, B. et al., Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. *J. Immunol.* 2011. **187**: 2222–2232.
- 179 Flanagan, R. S. and Heinrichs, D. E., A fluorescence based-proliferation assay for the identification of replicating bacteria within host cells. *Front Microbiol* 2018. **9**: 3084.
- 180 Colbert, J. D., Cruz, F. M. and Rock, K. L., Cross-presentation of exogenous antigens on MHC I molecules. *Curr. Opin. Immunol.* 2020. **64**: 1–8.
- 181 Childs, E., Henry, C. M., Canton, J. and Reis e Sousa, C., Maintenance and loss of endocytic organelle integrity: mechanisms and implications for antigen cross-presentation. *Open Biol.* 2021. **11**: 210194.
- 182 Embgenbroich, M. and Burgdorf, S., Current Concepts of Antigen Cross-Presentation. *Front. Immunol.* 2018. **9**: 1643.
- 183 Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R. and Germain, R. N., Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity.* 1997. **6**: 715–726.
- 184 Hermans, I. F., Silk, J. D., Yang, J., Palmowski, M. J., Gileadi, U., McCarthy, C., Salio, N. et al., The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. *J. Immunol. Methods.* 2004. **285**: 25–40.
- 185 Quah, B. J., Wijesundara, D. K., Ransinghe, C. and Parish, C. R., Fluorescent target array killing assay: a multiplex cytotoxic T-cell assay to measure detailed T-cell antigen specificity and avidity in vivo. *Cytometry A.* 2012. **81**: 679–690.
- 186 Oehen, S. and Brduscha-Riem, K., Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. *J. Immunol.* 1998. **161**: 5338–5346.
- 187 Lehmann, C. H., Heger, L., Heidkamp, G. F., Baranska, A., Luhr, J. J., Hoffmann, A. and Dudziak, D., Direct Delivery of Antigens to Dendritic Cells via Antibodies Specific for Endocytic Receptors as a Promising Strategy for Future Therapies. *Vaccines (Basel).* 2016. **4**: 8.
- 188 Amon, L., Hatscher, L., Heger, L., Dudziak, D. and Lehmann, C. H. K., Harnessing the complete repertoire of conventional dendritic cell functions for cancer immunotherapy, *Pharmaceutics.* 2020. **12**: 1–83.
- 189 Amon, L., Lehmann, C. H. K., Heger, L., Heidkamp, G. F. and Dudziak, D., The ontogenetic path of human dendritic cells, *Mol. Immunol.* 2020. **120**: 122–129.
- 190 Soares, H., Waechter, H., Glaichenhaus, N., Mougneau, E., Yagita, H., Mizenina, O., Dudziak, D. et al., A subset of dendritic cells induces CD4<sup>+</sup> T cells to produce IFN- $\gamma$  by an IL-12-independent but CD70-dependent mechanism in vivo, *J. Exp. Med.* 2007. **204**: 1095–1106.
- 191 den Haan, J. M. M., Lehar, S. M. and Bevan, M. J., Cd8<sup>+</sup> but Not Cd8<sup>-</sup> Dendritic Cells Cross-Prime Cytotoxic T Cells in Vivo, *J. Exp. Med.* 2000. **192**: 1685–1696.
- 192 Schlitzer, A., McGovern, N., Teo, P., Zelante, T., Atarashi, K., Low, D., Ho, A. W. S. et al., IRF4 Transcription Factor-Dependent CD11b<sup>+</sup> Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine Responses, *Immunity.* 2013. **38**: 970–983.



- 193 Tussiwand, R., Everts, B., Grajales-Reyes, G. E., Kretzer, N. M., Iwata, A., Bagaitkar, J., Wu, X. et al., Klf4 Expression in Conventional Dendritic Cells Is Required for T Helper 2 Cell Responses. *Immunity*. 2015. **42**: 916–928.
- 194 Segura, E., Durand, M., Amigorena, S., Similar antigen cross-presentation capacity and phagocytic functions in all freshly isolated human lymphoid organ-resident dendritic cells, *J. Exp. Med.* 2013. **210**: 1035–1047.
- 195 Cohn, L., Chatterjee, B., Esselborn, F., Smed-Sørensen, A., Nakamura, N., Chalouni, C., Lee, B. C. et al., Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3 + dendritic cells at cross presentation, *J. Exp. Med.* 2013. **210**: 1049–1063.
- 196 Yu, C. I., Becker, C., Metang, P., Marches, F., Wang, Y., Toshiyuki, H., Banchereau, J. et al., Human CD141+ dendritic cells induce CD4+ T cells to produce type 2 cytokines., *J. Immunol.* 2014. **193**: 4335–4343.
- 197 Jongbloed, S. L., Kassianos, A. J., McDonald, K. J., Clark, G. J., Ju, X., Angel, C. E., Chen, C. J. J. et al., Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens., *J. Exp. Med.* 2010. **207**: 1247–12460.
- 198 Tel, J., Schreibelt, G., Sittig, S. P., Mathan, T. S. M., Buschow, S. I., Cruz, L. J., Lambeck, A. J. A. et al., Human plasmacytoid dendritic cells efficiently cross-present exogenous Ags to CD8+ T cells despite lower Ag uptake than myeloid dendritic cell subsets. *Am. J. Blood Res.* 2013. **121**: 459–467.
- 199 Nizzoli, G., Krietsch, J., Weick, A., Steinfeld, S., Facciotti, F., Gruarin, P., Bianco, A. et al., Human CD1c+ dendritic cells secrete high levels of IL-12 and potently prime cytotoxic T-cell responses. *Am. J. Blood Res.* 2013. **122**: 932–942.
- 200 Belk, J. A., Daniel, B., Satpathy, A. T., Epigenetic regulation of T cell exhaustion, *Nat. Immunol.* 2022. 848–860.
- 201 Piccioli, D., Tavarini, S., Borgogni, E., Steri, V., Nuti, S., Sammiceli, C., Bardelli, M. et al., Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Am. J. Blood Res.* 2007. **109**: 5371–5379.

**Abbreviations:** DAPI: 4',6-diamidino-2-phenylindole · ATP: Adenosine 5'-triphosphate disodium salt · APC: antigen-presenting cells · BMDC: bone marrow-derived DC · CFDA-SE: carboxyfluorescein diacetate succinimidyl ester · DIA: data-independent acquisition mode · DC: dendritic cells · DNaseI: Deoxyribonuclease I · DEG: Differentially expressed gene · cDC: conventional DC · ddH<sub>2</sub>O: double distilled water · ETC: electron transport chain · ECM: extracellular matrix · FCS: Fetal calf serum · FOV: field of view · FACS: fluorescence-activated cell sorting · FLT3L: Fms-like tyrosine kinase 3 ligand · GM-CSF: granulocyte-monocyte colony-stimulating factor · iNOS: inducible nitric oxide synthetase · IMDM: Iscove Modified Dulbecco Media · LFQ: label-free quantification · LDH: lactate dehydrogenase · LC: Langerhans cells · LPS: lipopolysaccharide · LC-MS: liquid chromatography-mass spectrometry · CO: lowest concentration · LN: lymph nodes · MHC: major histocompatibility complex · MMP: matrix metalloproteases · MEM: Minimum Essential Medium · NaPox: NeutrAvidin Horseradish Peroxidase conjugate · OVA: ovalbumin · OXPHOS: oxidative phosphorylation · OCR: oxygen consumption rate · PCA: principal component analysis · pDC: plasmacytoid DC · PEG: polyethylene glycol · QC: quality control · SDS: sodium dodecyl sulfate · SD: standard deviation

**Full correspondence:** Prof. Björn E. Clausen, Research Center for Immunotherapy (FZI), University Medical Center of the Johannes-Gutenberg University Mainz, Mainz, Germany  
e-mail: bclausen@uni-mainz.de

**Additional correspondence:** Dr. Thomas Hieronymus, Institute for Biomedical Engineering, Department of Cell Biology, RWTH Aachen University, Medical Faculty, Aachen, Germany  
e-mail: Thomas.Hieronymus@rwth-aachen.de

Received: 28/3/2022  
Revised: 25/10/2022  
Accepted: 26/10/2022