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TITLE:

Assessment of Lymphocyte Migration in an *Ex Vivo* Transmigration System.

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KEYWORDS:

CCL17 (C-C Motif Chemokine 17)/TARC (thymus and activation related chemokine), CCL22 (C-C Motif Chemokine 22)/MDC (Macrophage-derived chemokine), CCR4 (CC Chemokine Receptor 4), CD4 T cell (CD4+ T helper cell)/Th2 cell, ILC2: (Group 2 Innate Lymphoid Cell), OVA (Chicken Egg Ovalbumin)

SUMMARY:

In this protocol, lymphocytes are placed in the top chamber of a transmigration system, separated from the bottom chamber by a porous membrane. Chemokine is added to the bottom chamber, which induces active migration along a chemokine gradient. After 48 hours, lymphocytes are counted in both chambers to quantitate transmigration.

ABSTRACT

Herein, we present an efficient method that can be executed with basic laboratory skills and materials to assess lymphocyte chemokinetic movement in an *ex vivo* transmigration system. Group 2 innate lymphoid cells (ILC2) and CD4+ T helper cells were isolated from spleens and lungs of chicken egg ovalbumin (OVA)-challenged BALB/c mice. We confirmed the expression of CCR4 on both CD4+ T cells and ILC2, comparatively. CCL17 and CCL22 are the known ligands for CCR4; therefore, using this *ex vivo* transmigration method we examined CCL17- and CCL22-induced movement of CCR4+ lymphocytes. To establish chemokine gradients, CCL17 and CCL22 were placed in the bottom chamber of the transmigration system. Isolated lymphocytes were then added to top chambers and over a 48-hour period the lymphocytes actively migrated through 3 μ m pores towards the chemokine in the bottom chamber. This is an effective system for determining the chemokinetics of lymphocytes, but, understandably, does not mimic the complexities found in the *in vivo* organ microenvironments. This is one limitation of the method that can be overcome by the addition of *in situ* imaging of the organ and lymphocytes under

study. In contrast, the advantage of this method is that it can be performed by an entry-level technician at a much more cost-effective rate than live imaging. As therapeutic compounds become available to enhance migration, as in the case of tumor infiltrating cytotoxic immune cells, or to inhibit migration, perhaps in the case of autoimmune diseases where immunopathology is of concern, this method can be used as a screening tool. In general, the method is effective if the chemokine of interest is consistently generating chemokinetics at a statistically higher level than the media control. In such cases, the degree of inhibition/enhancement by a given compound can be determined as well.

INTRODUCTION:

This original transmigration method was presented by Stephen Boyden in 1962 in the *Journal of Experimental Medicine*¹. Much of what we know about chemotaxis and chemokinetics would not be possible without the development of the Boyden chamber. Prior to the discovery of the first chemokine in 1977, ex vivo transmigration systems were used to learn about serum-factors that could arrest cellular movement in macrophages while amplifying cellular motility in neutrophils^{1,2}. A massive wealth of knowledge has been developed regarding immune cell migration, and to date, 47 chemokines have now been discovered with 19 corresponding receptors^{3,4}. In addition, multitudes of inhibitors/enhancers of these chemokine pathways have undergone development for therapeutic purposes⁵⁻⁸. Many of those compounds have been tested in similar transmigration chambers to understand direct interactions between the compounds and immune cell responsiveness to a given chemokine⁹.

Transmigration, or diapedesis, into inflamed tissue is an essential process to a healthy inflammatory response to clear infection^{10,11}. A Boyden chamber, transmigration system, or transwell apparatus are generally composed of two chambers separated by a porous membrane^{1,12}. The bottom chamber most often holds media containing the chemokine of interest, while leukocytes are placed in the top chamber. The size of the pore in the membrane can be selected based on the size of the cell of interest. For this project, we selected a 3 μm porous membrane, as lymphoid cells are 7-20 μm in size, depending on the stage of cellular development. This pore size ensures that these cells are not passively falling through the pores, but that they are actively migrating in response to the chemokine gradient.

The major advantage of this protocol is its cost effectiveness. In vivo transmigration is difficult because it requires extensive training in animal handling and surgery, and often involves high-powered microscopy that is not always available to a researcher. Cost effective screening of compounds thought to enhance or inhibit transmigration can be accomplished in advance of in vivo imaging. Because the transmigration system is tightly controlled, cells may be treated initially then added to the transwell apparatus, or, vice versa, the chemokine may be treated first with a chemokine inhibitor then cells added to the transwell apparatus. Lastly, endothelial cells and/or basement membrane proteins can be added to the bottom of the transwell insert 1-2 days prior to the transmigration experiment to understand the involvement of these barrier cells in chemokinetics. Again, these manipulations of the system provide a powerful means of determining important information about the effectiveness of a given compound in advance of more complicated in vivo studies.

Utilizing a transmigration chamber system is an effective way to assess lymphocyte mobility under various in vivo and in vitro conditions¹²⁻¹⁴. Herein, we describe an optimized method for assessing ex vivo lymphocyte responsiveness to chemokines in a transmigration chamber. In this example experiment, CD4+ T cells and group 2 innate lymphoid cells (ILC2) were isolated from male and female, BALB/c mice following OVA-allergen exposure. A hypothesis was generated that CCR4+ CD45+ Lineage- (LIN-) ILC2 from allergen-challenged mice would migrate more efficiently towards CCL17 and CCL22 than CCR4+ CD4+ T helper cells. CCL17 and CCL22 are chemokines commonly produced by dendritic cells and macrophages of the M2 (allergic) phenotype, among other cells, in allergy^{15,16}. CCL17 and CCL22 can be thought of as biomarkers of allergic inflammation as they are readily detected in the lungs during airway exacerbations¹⁶⁻¹⁸. Importantly, CCR4 expression is elevated in comparison to untreated controls, as revealed in bioinformatic data generated from ILC2 isolated from house dust mite treated animals, and similarly ILC2 from naïve animals treated ex vivo with IL-33 (allergen-promoting innate cytokine) upregulates CCR4^{19,20}. Furthermore, according to data for ILC2 in the Immunological Genome Project database (www.immgen.org), CCR4 mRNA is highly expressed in these innate immune cells. To date, little is known regarding trafficking of ILC2 into tissues, but it is likely that the ILC2 and CD4+ T cells use similar chemokines and receptors for chemotaxis and chemokinetics as they express similar transcription factors and receptors. Thus, we compared CCL17 versus CCL22 responsiveness, of ILC2 and CD4+ T lymphocytes, from both male and female, OVA-challenged animals.

PROTOCOL:

All methods described here were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Nebraska Medical Center (UNMC) and the University of Utah.

1. Setup and Preparation of Reagents

1.1. Prepare complete RPMI (Roswell Park Memorial Institute) media.

1.1.1. Add 10 mL of heat-inactivated fetal bovine serum (FBS) to 90 mL of RPMI.

1.1.2. Add 1 mL of 100x Penicillin-Streptomycin-Glutamine to 100 mL of 10% FBS RPMI.

1.2. Prepare ILC2 Expansion Media.

1.2.1. Add IL-2 and IL-33 (20 ng/mL each cytokine) to 10 mL of complete RPMI.

1.2.2. If stock cytokines are 10 µg/mL, pipette 20 µL of IL-2 and IL-33 into a 15 mL tube containing 10 mL of complete RPMI media.

1.3. Prepare Lung Dissociation Medium.

133
134 1.3.1. Add 50 mg of type 1 collagenase to 250 mL of unsupplemented RPMI.

135
136 1.3.2. Add 2.5 mL of 100x penicillin-streptomycin-glutamine to the 250 mL of media in step 1.3.1.

137
138 1.3.3. Gently mix the media to ensure the type 1 Collagenase is completely dissolved before use.

139
140 1.4. Prepare Serum-free RPMI.

141
142 1.4.1. Dilute 1 g of lyophilized bovine serum albumin (BSA) in 200 mL of RPMI.

143
144 1.4.2. Add 2 mL of 100x penicillin-streptomycin-glutamine.

145
146 1.4.3. Gently mix the media to ensure the BSA is completely dissolved in the media before use.

147
148 1.5. Prepare Migration Medium with CCL17.

149
150 1.5.1. Acquire 10 mL of serum-free RPMI and add CCL17 [50 ng/mL].

151
152 1.5.1.1. If stock CCL17 is 10 µg/mL, add 50 µL of CCL17 stock to 10 mL of serum-free RPMI media.

153
154 1.6. Prepare Migration Medium with CCL22.

155
156 1.6.1. Acquire 10 mL of serum-free RPMI and add CCL22 [50 ng/mL].

157
158 1.6.2. If stock CCL22 is 10 µg/mL, add 50 µL of CCL22 stock to 10 mL of serum-free RPMI media.

159
160 1.7. Prepare CCR4 Antibody Staining Cocktail.

161
162 1.7.1. For 10 tests total, add 5 µL of each of the following antibodies to a 5 mL tube: anti-mouse
163 CCR4, CD19, CD11b, CD45, ST2, and ICOS.

164
165 NOTE: Example of how to make antibody cocktail for 10 samples: 0.5 µL of each antibody x 10
166 samples = 5 µL of each of the antibodies listed in 1.7.1.

167
168 1.7.2. For 10 tests total, add 2.5 µL of the following antibodies to the antibody cocktail from step
169 1.7.1: anti-mouse CD3, CD11c, and NK1.1.

170
171 NOTE: Example to complete the antibody cocktail for 10 samples: 0.25 µL x 10 samples = 2.5 µL
172 of CD3, CD11c and NK1.1 antibodies.

173
174 1.7.3. Store the CCR4 Antibody Staining Cocktail at 4 °C until ready to add to the samples. Discard
175 antibody cocktail after 1 week if not used.

176

177 1.8. Prepare 1x Stabilizing Fixative.

178
179 1.8.1. Add 10 mL of deionized-distilled water to 5 mL of 3x stabilizing fixative concentrate

180
181 **2. Preparation of allergen-challenged BALB/c mice**

182
183 NOTE: Male and female BALB/c mice were purchased from Charles River (UNMC) or Jackson
184 Laboratories (University of Utah) at 6 to 8 weeks of age.

185
186 2.1. After acclimation (1 week), sensitize all animals to OVA.

187
188 2.1.1. Combine 100 µg/mL OVA adsorbed to aluminum hydroxide (20 mg/mL) in a 5 mL
189 polystyrene tube.

190
191 2.1.2. Mix the tube and immediately draw 500 µL of the OVA-alum suspension into a 1 mL, 28 G
192 syringe (insulin syringe).

193
194 2.1.3. Place a mouse in a bell jar containing isoflurane (1–2 mL under a false floor, so that the
195 animal is not standing directly in isoflurane). Allow the mouse to go under anesthesia for
196 approximately 1–2 min, or until the rate of respiration drops.

197
198 2.1.4. Quickly pick up the mouse by the fur on the back and shoulders and inject 100 µL of OVA-
199 alum intraperitoneally per mouse^{21,22}.

200
201 2.1.5. Place the mouse back in its cage and watch to make sure they regain mobility; this should
202 occur within 2–5 min.

203
204 2.2. Seven days after sensitization, subject all animals to intranasal (i.n.) 1.5% OVA diluted in
205 sterile saline in a nebulization chamber (Data Sciences International) for 20 min.

206
207 2.2.1. Remove mice from their cages and place them in the animal nebulization chamber. Close
208 the lid on the chamber.

209
210 2.2.2. Attach the nebulization hose to the input spout on the nebulization chamber.

211
212 2.2.3. Add 30 mL of 1.5% OVA diluted in sterile saline to the nebulization cup on the nebulizer.

213
214 2.2.4. Turn on the nebulizer and allow the chamber to fill with mist for 20 min.

215
216 2.2.5. Turn off the nebulizer and let the mist settle.

217
218 2.2.6. Return animals to their cages.

219
220 2.3. Repeat step 2.2 for a total of 5 times, on 5 consecutive days, to induce allergic inflammation.

3. Isolation of CD4⁺ T cells from spleens and lungs of OVA-challenged mice

3.1. Humanely euthanize all OVA-treated male and female animals by CO₂ asphyxiation according to approved IACUC protocols, using 2 to 3 animals per group, per experiment.

3.2. Excise lungs and spleens from animals and place tissues in separate dissociation tubes based on the tissue type and sex of the animal²³.

3.3. Dissociate lung tissue in 500 µL of Lung Dissociation media (25 U/mL; collagenase, type 1) in the automated tissue dissociator using the 'lung' protocol.

3.3.1. Repeat 3.2 and 3.3 a total of two times.

3.4. Dissociate spleen tissue in 500 µL of complete RPMI media using the 'spleen' protocol on the automated tissue dissociator.

NOTE: The remaining steps should be performed in a Biological Safety cabinet using sterile technique.

3.5. Rinse the dissociation tubes containing lung and spleen homogenates with 5 mL of additional Lung Dissociation media or Complete RPMI, respectively.

3.6. Filter cell suspensions through a 40 µm cell strainer and collect into 50 mL conical tubes.

3.7. Incubate lung homogenates for 15–30 min in a 37 °C incubator with 5% CO₂ to further dissociate lung tissue.

3.8. Add 5 mL of Complete RPMI to the lung and spleen homogenates and pellet the cells at the bottom of the 50 mL tubes using centrifugation; 378 x g at room temperature (RT) for 5 min.

3.9. Combine splenocytes and lung cells into a single 50 mL conical tube and determine total cell counts using the automated cell counter.

3.10. Adjust male and female cell suspensions to 1 X 10⁸ cells/mL in separation buffer and add to a 5 mL polystyrene tube.

NOTE: The Enrichment Protocol can be adjusted up to 14 mL polystyrene tubes when more cells are acquired from spleen and lungs. This protocol was designed for tissues from 2–3 mice per group; therefore a 5 mL tube should suffice.

3.11. Use approximately two thirds of the total cells for ILC2 isolation according to the ILC2 enrichment protocol.

3.11.1. Add antibody cocktail (50 μ L/mL) to the cell suspension and incubate for 5 minutes at RT.

3.11.2. Vortex rapid spheres for 30 s and add to the sample at a rate of 75 μ L/mL of cell suspension. Gently mix and incubate for 5 min at RT.

3.11.3. Top the tube up to 3 mL total volume with separation buffer and place in the 8-chamber easy separation magnet. Incubate for 3 minutes at RT.

3.11.4. Tip the magnet forward (away from the sphere-antibody-cell complexes adhered to the back of the tube) and pipette off the cell suspension into a clean 5 mL tube.

3.11.5. Add 1.5 mL of complete RPMI to the tubes and centrifuge at 378 x *g* for 5 min at RT.

3.11.6. Pour off the media from the cell pellet and resuspend the ILC2 at 1 x 10⁷ cells per mL.

3.11.7. Place 100 μ L of male and female ILC2 per well in a U-bottom, 96-well plate and add 100 μ L of ILC2 Expansion Media to each well.

3.11.8. Incubate the cells for 4–5 days to expand the ILC2.

3.11.9. Collect the ILC2 into a 5 mL tube and add up to 4.5 mL of serum-free RPMI. Centrifuge the cells at 378 x *g* for 5 min at RT.

3.11.10. Count cells using a hemacytometer and resuspend at 1 x 10⁷ ILC2 per mL in serum-free RPMI.

3.12. Use the remaining cells for the CD4⁺ T cell isolation procedure, which is conducted according to the mouse CD4⁺ T cell isolation protocol with few modifications.

3.12.1. Add rat serum (50 μ L/mL) to the CD4 T cell enrichment suspension.

3.12.2. Add Isolation cocktail (50 μ L/mL) to the sample and incubate for 10 min at RT.

3.12.3. Vortex rapid spheres for 30 s and add to the sample at a rate of 75 μ L/mL.

3.12.4. Gently mix the cell suspension and incubate for 2.5 min and RT

3.12.5. Top the samples up to 3 mL and place the 5 mL tubes into the 8-chamber easy separation magnet and incubate for 5 min at RT.

3.12.6. Tip the magnet forward and pipette the cell suspension into a clean 5 mL tube.

3.12.7. Add 1.5 mL of serum-free RPMI to the tubes and centrifuge at 378 x *g* for 5 min at RT.

3.12.8. Pour off the media from the cell pellet and resuspend the CD4⁺ T cells at 1×10^7 cells per mL in serum-free RPMI.

4. Determine CCR4 expression on CD4⁺ T cells and group 2 innate lymphoid cells (ILC2) from OVA-challenged animals by flow cytometry.

NOTE: The following steps may be performed on an open bench top as they are non-sterile techniques.

4.1. Acquire approximately $1\text{--}2.5 \times 10^5$ ILC2 cells from step 3.11.10 and $1\text{--}2.5 \times 10^6$ CD4⁺ T cells from step 3.12.8 in separate 5 mL tubes.

4.1.1. Keep an additional tube of at least 5.0×10^4 CD4⁺ T cells as an unstained control.

4.2. Suspend the cells in 100–200 μ L of FACS buffer and add 1 μ L of Fc Block to every tube, then incubate on ice (or in a 4 °C refrigerator) for 10 min.

4.3. Add 1–2 mL of FACS Buffer to every tube and centrifuge at $378 \times g$ for 5 min at RT.

4.4. Pour off the supernatant and resuspend the cells in 100–200 μ L of FACS Buffer.

4.5. Add 37.5 μ L of the CCR4 Antibody Staining Cocktail to the cell suspension in every tube except the tube containing the ‘unstained’ cells.

4.6. Incubate the tubes on ice, or in a 4 °C refrigerator, for 20–30 minutes.

4.7. Add 1–2 mL of FACS Buffer to every tube and centrifuge at $378 \times g$ for 5 min at RT.

4.8. Pour off the supernatant.

4.9. Repeat steps 4.7 and 4.8.

4.10. Add 250–300 μ L of 1x stabilizing fixative (see **Table of Materials**) to every tube.

4.11. Prepare single-colored bead controls for each antibody in the CCR4 antibody cocktail according to the protocol provided with the compensation beads.

4.11.1. Vortex the compensation beads.

4.11.2. Add one drop of the beads to each single-colored control tube.

4.11.3. Add 1 μ L of each antibody in the CCR4 Antibody cocktail to its own labeled tube.

4.11.4. Gently mix and incubate for 10 min in a 4 °C refrigerator or on ice.

4.11.5. Add 1–2 mL of FACS Buffer to all the tubes and centrifuge at $378 \times g$ for 5 min at RT.

4.11.6. Pour off the supernatant and resuspend the beads in 200 μL of FACS buffer.

4.11.7. Refrigerate the single-colored controls until all the samples are stained and ready to be analyzed on the flow cytometer.

4.12. Analyze the unstained cells, single-colored controls and the experimental samples on the flow cytometer within 24 h of fixation.

5. Ex vivo transmigration procedure

NOTE: The following steps should be performed in a Biological Safety Cabinet, as they require sterile technique.

5.1. Acquire the ILC2 from step 3.11.10 and CD4 T cells from step 3.12.8 and determine the number of transwell inserts needed for the experiment.

NOTE: Example: For 1.2 mL of enriched CD4 T cells from step 3.12.8, multiply $1.2 \times 1000 \mu\text{L} = 1200 \mu\text{L}$; then divide 1200 μL by $100 \mu\text{L} = 12$, the number of inserts needed for CD4 T transmigration.

5.2. Gently move the 3 μm transwell inserts from the middle rows of a 24-well plate.

5.3. Add 500 μL of migration media with CCL17 to approximately one-third of the wells.

5.4. Add 500 μL of migration media with CCL22 to another one-third of the wells.

5.5. Add 500 μL of serum-free RPMI containing no chemokine to the last one-third of the wells.

5.6. Clearly label the lid on the plate with the appropriate transmigration media placed in the bottom wells.

5.7. Place the transwell inserts back into the wells containing the various treatments.

5.8. Gently add 100 μL of CD4 T cells or ILC2 to the top well of each insert. Do not mix or pipette the cell suspension up and down in the transwells, as this may confound the results of the experiment.

5.9. Clearly label the lid of the plate with the cell type placed in each well and write the date and time of day that the cells were added.

5.10. Repeat steps 5.2 to 5.9 until all the cells have been placed in transwell inserts with media.

5.11. Gently place the plate in a 37 °C incubator with 5% CO₂ for 48 hours. Minimize contact with the plate over the incubation period.

6. Quantification of ex vivo transmigration

6.1. Gently remove the plate from the incubator and remove all the transwell inserts from the middle rows into the empty wells just above or below.

6.2. Collect the cells from the bottom and top wells of the transwell inserts into tubes labeled with TOP or BOTTOM, with CCL17, CCL22, or media, with the cell type, and with the replicate number (at least 3 replicates per experiment).

6.3. Rinse the bottom wells with 500 µL of 1X PBS and collect this rinse into the corresponding tube.

6.4. Rinse the top wells with 250 µL of 1X PBS and collect this rinse into the corresponding tube.

6.5. Pellet the cells by centrifugation at 378 x g for 5 min at RT.

6.6. Gently pipette off all supernatant from the cell pellet.

6.7. Resuspend T cells and ILC2 into 50 µL of 1x PBS.

6.8. Take 10 µL of the cell suspensions and add to 90 µL of 0.4% trypan blue.

6.9. Count the cells on the automated cell counter.

6.9.1. Record %viability.

6.9.2. Record the cell count per mL for each sample.

6.9.3. Determine the total number of cells per treatment in the top and the bottom chamber; record the cell counts.

REPRESENTATIVE RESULTS:

CCR4 expression on CD4+ T cells and ILC2.

For the success of the *ex vivo* transmigration experiment, it is imperative to determine whether the lymphocytes are responsive to CCL17 and CCL22 through CCR4; therefore, we determined CCR4 expression on both CD4+ T cells and ILC2 by flow cytometry. While it is well known that OVA-specific CD4+ helper T cells express CCR4, less is known of the expression of CCR4 on ILC2. **Figure 1** shows representative results of CCR4 expression, comparatively, on CD4+ T cells (**Figure 1A,C**) and ILC2 (**Figure 1B,D**) from male and female, OVA-challenged BALB/c mice. Flow cytometry was used to detect CCR4 using a monoclonal antibody conjugated to allophycocyanin

(APC). Using One-Way Analysis of Variance (ANOVA), we determined there were no differences in CCR4 expression between male and female hosts (**Figure 1A–D**), however, the expression of CCR4 on a per cell basis (MFI) on ILC2 was higher in comparison to CD4+ T cells (**Figure 1C** compared to **Figure 1D**). These results are important in showing that the ILC2 and CD4+ T cells should respond to CCL17 and CCL22 in the following experiment.

Responsiveness of CD4+ T cells to CCR4 ligands in the top and bottom chambers of a transmigration system.

CD4+ T cells from male, OVA-challenged BALB/c mice were isolated from the lungs and spleens and placed in the top chamber of a transmigration apparatus separated by a 3 μ M porous membrane (**Figure 2**). A summary of the in vivo preparation of OVA-treated mice (**Figure 2A**) and the transmigration procedure (**Figure 2B**) are shown for reference. A combination of CCL17 (25 ng/mL) and CCL22 (25 ng/mL) were placed in the top chamber, the bottom chamber or both the top and bottom chambers to confirm (**Figure 2C**), (1) that the CD4+ T cells from OVA-challenged animals were responsive to CCR4 ligands, and (2) that chemokine-induced migration was an active process by which T cells were moving through the pores in response to the chemokine gradient, and that the lymphocytes were not moving through the pores independent of chemokine. A media (No Chemokine) control was included to show that CD4+ T cells could not migrate through the 3 μ M pores without stimulation. In this condition, the highest percentage of cells remained in the top chamber. When the chemokines were placed in the top and bottom chamber simultaneously, we detected 52% of the total T cells in the bottom chamber and 48% of the cells in the top chamber (TOP/BOTTOM treatment). As expected, the distribution of cells moved in response to chemokine placed only in the top or only in the bottom chamber, as we detected the highest percentage of cells in the compartment where chemokine was present.

Responsiveness of CD4+ T cells and ILC2 to CCL17 and CCL22 in an ex vivo transmigration apparatus.

CD4 T cells and ILC2 from male and female, OVA-challenged mice were isolated from lungs and spleens then placed in the top chamber of a transwell transmigration apparatus (**Figure 3**). The bottom chamber of the apparatus was filled with untreated cell culture media, media containing CCL17, or media containing CCL22. The representative results show that less than 14% ($13.37 \pm 6.5\%$) of the cells migrated in media control conditions (**Figure 3A–D**). In response to CCL22, both cell types, regardless of whether they were from male or female hosts, responded to CCL22 (**Figure 3A–D**), however, the results for CCL17 were less consistent. CCL17 only induced significant migration for the female CD4 T cells and ILC2 in comparison to media alone (**Figure 3C,D**). CCL17 treatment was not different than media for male CD4+ T cells or male ILC2 (**Figure 3A,B**), and CCL22 induced greater migration than CCL17 in male ILC2 (**Figure 2B**).

Suboptimal transmigration results for CD4+ T cells with low viability.

Suboptimal results were generated to provide the researcher an example of what to expect when the transmigration experiment does not work properly (**Figure 4**). We isolated male CD4+ T cells from animals according to this protocol and placed them in the top well of transmigration system. After the CD4+ T cells were added, however, the plate remained at room temperature for the first 24 hours, then the plate was moved into the incubator for the remaining 24 hours of the

incubation period. Not surprisingly, we detected no migration towards CCL17 and CCL22 (**Figure 4A**) and the viability of the cells was notably low (< 15%) for the cells in the top (**Figure 4B**). These flawed results highlight the importance of using the correct temperatures and conditions detailed in this protocol to achieve optimum results.

FIGURE LEGENDS:

Figure 1. CCR4 expression on CD4+ T cells and ILC2. 7- to 9-week old, male and female, BALB/c mice were injected once with 100 μ L of OVA-adsorbed to aluminum hydroxide (500 μ g/mL; OVA and 20 mg/mL aluminum hydroxide) 7 days prior to the first of 5, repetitive, daily airway challenges with 1.5% OVA in saline. Allergen-challenged animals were humanely euthanized, and lung and spleen tissue was collected for ILC2 and CD4+ T cells isolation. A small aliquot of cells was then stained and analyzed by flow cytometry to determine the level of CCR4 on each cell type. **(A)** Frequency of CD4+ T cells that are CCR4+ from OVA+ mice, where the error bars represent the standard error of the mean (+ SEM). **(B)** Frequency of ILC2 that were CCR4+ (+SEM). **(C, D)** Mean fluorescence intensity (+SEM) of CCR4 on **(C)** CD4+ T cells and **(D)** ILC2. A total of 13 mice were used to generate these data, and the flow experiment was repeated two times, with 3 replicates of each treatment per experiment. Significance was determined by One-Way ANOVA; n.d. indicates there were no differences between groups.

Figure 2. Responsiveness of CD4+ T cells to CCR4 ligands in the top and bottom chambers of a transmigration system. Male BALB/c mice sensitized and challenged with chicken egg ovalbumin (OVA) and CD4+ T cells were isolated from the spleens and lungs **(A, B)**. For this transmigration experiment, CD4+ T cells were suspended in serum-free media at 1×10^7 cells/mL. CCL17 and CCL22 were added to serum-free media at a concentration of 50 ng/mL (25 ng/mL of each chemokine to achieve a total of 50 ng/mL). Chemokine-containing media was added to the top chamber only, to the bottom chamber only, or to both the top and bottom chambers. A total volume of 500 μ L of transmigration media was added to the bottom wells and 100 μ L of cellular suspension (1×10^6 cells/well) was added to the top well. Transmigration was measured after 48 hours in culture **(C)**. These data were generated from a single experiment, 3 OVA-treated, male mice were used for tissue collection, and 3 replicates were made per treatment. Statistical significance was determined by One-Way ANOVA; * $p < 0.05$.

Figure 3. Responsiveness of CD4+ T cells and ILC2 to CCL17 and CCL22 in an *ex vivo* transmigration apparatus. Mice were prepared as in **Figure 1** for CD4+ T cell and ILC2 isolation from spleens and lungs. CD4+ T cells and ILC2 were suspended in serum-free media at 1×10^7 cells/mL. CCL17 or CCL22 were added to serum-free media at a concentration of 50 ng/mL. 500 μ L of transmigration media was added to the bottom wells and 100 μ L of cellular suspension (1×10^6 cells/well) was added to the top well. Transmigration was measured after 48 h in culture. **(A)** CD4+ T cells and **(B)** ILC2 from male hosts were treated with media as control, CCL17, or CCL22. Similarly, **(C)** female CD4+ T cells and **(D)** female ILC2 were treated with media, CCL17, or CCL22. A total of 14 mice were used to generate these data. The transmigration experiment was repeated 4 times, with 3–6 replicates of each treatment per experiment. Significance was determined by One-Way ANOVA; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 4. Suboptimal transmigration results for CD4 T cells with low viability. Naïve male BALB/c mice were acquired for lung and spleen tissue collection and CD4+ T cell isolation as in **Figure 1**, **Figure 2**, and **Figure 3**. CD4 T cells were added to the top chamber of the transmigration apparatus and serum-free media containing CCL17, CCL22 or no chemokine (media control) were added to the bottom well. For the first 24 h of the experiment the plate was left at room temperature, then it was moved to a 37 °C incubator with 5% CO₂ for an additional 24 h. **(A)** Percentage of cells remaining in the top and bottom wells after 24 hours. **(B)** Viability of the CD4+ T cells in the top and bottom chamber following poor incubation conditions. These data were generated from a single experiment, 3 naïve male mice were used for tissue collection, and 3 replicates were made per treatment. Statistical significance was not determined.

DISCUSSION:

Herein, we present a well-established method for assessing chemokine-induced migration of lymphocytes in an ex vivo transmigration system. There are several critical steps in the protocol, the first of which is verifying the expression of the correct chemokine receptor on the immune cells in the experiment. In our hands, we chose CCR4 because of the body of literature that highlights the importance of CCR4 on Th2 helper T cells in allergic inflammation. Ovalbumin-induced inflammation was shown previously to be limited by at least two CCR4 antagonists^{24,25}; however, this was prior to the discovery of the group 2 innate lymphoid cells (ILC2)^{26,27}. We generated novel data showing that ILC2 cells express higher CCR4 than CD4+ T cells and showed that these cells were consistently responsive to CCL22.

A second critical step to follow in the protocol is to ensure that cells are kept in optimum medium for culture prior to beginning the transmigration part of the protocol. In the case of ILC2, we had to culture these cells in ILC2 Expansion Media that contains both IL-2 and IL-33. IL-2 and IL-7 are both reported in the literature to support ILC2 in culture for up to 14 days^{28,29}. If viability becomes an issue for CD4+ T cells and ILC2 in future experiments, the addition of IL-2 or IL-7 would likely improve survival of the lymphocytes until the endpoint of the experiment. Each of the media presented herein were defined over the course of several experiments and were optimized for use in this protocol^{14,30,31}. In **Figure 4**, we presented faulty results to demonstrate the importance of using an incubator with proper temperature and 5% CO₂. Keeping the transmigration plates in the incubator where they will not be disturbed is another critical step for the success of the protocol.

As stated previously, there are advantages to using the in vivo microscopy available at most institutions, however, in vivo imaging can be time consuming and costly. An alternative experimental procedure that is less costly uses microfluidics in combination with chemokine gradients to understand leukocyte extravasation and tissue migration³²⁻³⁴. These systems hold scientific value because they assess the complexities of cell kinetics that involves endothelial cells, which can be grown onto the capillaries of the microfluidic systems. Furthermore, these microfluidic systems assess the importance of adherences proteins (e.g., E-cadherin) on the endothelial cells and integrins on the immune cells in the process of cell adherence under blood flow. Nonetheless these systems require specialized equipment and complex computational

programming and statistics to determine the importance of each treatment conditions. Therefore, although the limitation of the transmigration method presented here is that it is artificial in nature, it can be used as an important screening tool to limit the waste of unnecessary reagents in subsequent in vivo methods. The significance of the method is that as new cells are discovered, as is the case for ILC2, we can screen these cells for their responsiveness to known chemokines. This is one of the future applications involving ILC2 and potential therapies that may inhibit their migration into the lungs during asthma exacerbation. This transmigration protocol will be used to screen various inhibitors that may be used to limit CCR4 or other chemotactic mediators involved in recruiting ILC2. Altogether, this ex vivo transmigration protocol will lead to the generation of critical data that can be verified with future in vivo experiments.

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REFERENCES:

- 1 Boyden, S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *The Journal of Experimental Medicine*. **115**, 453-466 (1962).
- 2 Moser, B. Editorial: History of Chemoattractant Research. *Frontiers in Immunology*. **6**, 548 (2015).
- 3 Borroni, E. M., Savino, B., Bonecchi, R., Locati, M. Chemokines sound the alarmin: The role of atypical chemokine in inflammation and cancer. *Seminars in Immunology*. **38**, 63-71 (2018).
- 4 Charo, I. F., Ransohoff, R. M. The many roles of chemokines and chemokine receptors in inflammation. *The New England Journal of Medicine*. **354** (6), 610-621 (2006).
- 5 Abboud, D., Hanson, J. Chemokine neutralization as an innovative therapeutic strategy for atopic dermatitis. *Drug Discovery Today*. **22** (4), 702-711 (2017).
- 6 Aldinucci, D., Casagrande, N. Inhibition of the CCL5/CCR5 Axis against the Progression of Gastric Cancer. *International Journal of Molecular Sciences*. **19** (5), pii: E1477 (2018).
- 7 Chonco, L. et al. Novel DNA Aptamers Against CCL21 Protein: Characterization and Biomedical Applications for Targeted Drug Delivery to T Cell-Rich Zones. *Nucleic Acid Therapy*. **28** (4), 242-251 (2018).
- 8 Trivedi, P. J., Adams, D. H. Chemokines and Chemokine Receptors as Therapeutic Targets in Inflammatory Bowel Disease; Pitfalls and Promise. *Journal of Crohn's and Colitis*. **12** (12), 1508 (2018).

617 9 Pietrosimone, K. M., Bhandari, S., Lemieux, M. G., Knecht, D. A., Lynes, M. A. In vitro
 618 assays of chemotaxis as a window into mechanisms of toxicant-induced
 619 immunomodulation. *Current Protocols in Toxicology*. **58** (Unit 18.17) (2013).
 620 10 Davis, D. M. How studying the immune system leads us to new medicines. *Lancet*. **391**
 621 (10136), 2205-2206 (2018).
 622 11 Culley, F. J., Pennycook, A. M., Tregoning, J. S., Hussell, T., Openshaw, P. J. Differential
 623 chemokine expression following respiratory virus infection reflects Th1- or Th2-biased
 624 immunopathology. *Journal of Virology*. **80** (9), 4521-4527 (2006).
 625 12 Denney, H., Clench, M. R., Woodroffe, M. N. Cleavage of chemokines CCL2 and CXCL10
 626 by matrix metalloproteinases-2 and -9: implications for chemotaxis. *Biochemical and*
 627 *Biophysics Research Communications*. **382** (2), 341-347 (2009).
 628 13 Burrell, B. E. et al. Lymph Node Stromal Fiber ER-TR7 Modulates CD4+ T Cell Lymph Node
 629 Trafficking and Transplant Tolerance. *Transplantation*. **99** (6), 1119-1125 (2015).
 630 14 Warren, K. J., Iwami, D., Harris, D. G., Bromberg, J. S., Burrell, B. E. Laminins affect T cell
 631 trafficking and allograft fate. *The Journal of Clinical Investigation*. **124** (5), 2204-2218
 632 (2014).
 633 15 Hirata, H. et al. Th2 cell differentiation from naive CD4(+) T cells is enhanced by autocrine
 634 CC chemokines in atopic diseases. *Clinical and Experimental Allergy: Journal of the British*
 635 *Society for Allergy and Clinical Immunology*. 10.1111/cea.13313 (2018).
 636 16 Lin, R., Choi, Y. H., Zidar, D. A., Walker, J. K. L. beta-Arrestin-2-Dependent Signaling
 637 Promotes CCR4-mediated Chemotaxis of Murine T-Helper Type 2 Cells. *American Journal*
 638 *of Respiratory Cell and Molecular Biology*. **58** (6), 745-755 (2018).
 639 17 Zhang, Y. et al. A new antagonist for CCR4 attenuates allergic lung inflammation in a
 640 mouse model of asthma. *Science Reports*. **7** (1), 15038 (2017).
 641 18 Lu, Y. et al. Dynamics of helper CD4 T cells during acute and stable allergic asthma.
 642 *Mucosal Immunology*. **11** (6), 1640-1652 (2018).
 643 19 Li, B. W. S., Beerens, D., Brem, M. D., Hendriks, R. W. Characterization of Group 2 Innate
 644 Lymphoid Cells in Allergic Airway Inflammation Models in the Mouse. *Methods in*
 645 *Molecular Biology*. **1559**, 169-183 (2017).
 646 20 Li, B. W. S. et al. Group 2 Innate Lymphoid Cells Exhibit a Dynamic Phenotype in Allergic
 647 Airway Inflammation. *Frontiers in Immunology*. **8**, 1684 (2017).
 648 21 Warren, K. J. et al. Sex differences in activation of lung-related type-2 innate lymphoid
 649 cells in experimental asthma. *Annals of Allergy, Asthma, Immunology: Official Publication*
 650 *of the American College of Allergy, Asthma, Immunology*. 10.1016/j.anai.2016.11.011
 651 (2016).
 652 22 Warren, K. J. et al. Ovalbumin-sensitized mice have altered airway inflammation to
 653 agriculture organic dust. *Respiratory Research*. **20** (1), 51 (2019).
 654 23 Poole, J. A. et al. alphabeta T cells and a mixed Th1/Th17 response are important in
 655 organic dust-induced airway disease. *Annals of Allergy, Asthma, Immunology: Official*
 656 *Publication of the American College of Allergy, Asthma, Immunology*. **109** (4), 266-273
 657 e262 (2012).
 658 24 Matsuo, K. et al. A CCR4 antagonist ameliorates atopic dermatitis-like skin lesions induced
 659 by dibutyl phthalate and a hydrogel patch containing ovalbumin. *Biomedical*
 660 *Pharmacotherapy*. **109**, 1437-1444 (2019).

661 25 Mikhak, Z. et al. Contribution of CCR4 and CCR8 to antigen-specific T(H)2 cell trafficking
662 in allergic pulmonary inflammation. *The Journal of Allergy and Clinical Immunology*. **123**
663 (1), 67-73 e63 (2009).

664 26 Monticelli, L. A. et al. Innate lymphoid cells promote lung-tissue homeostasis after
665 infection with influenza virus. *Nature Immunology*. **12** (11), 1045-1054 (2011).

666 27 Saenz, S. A. et al. IL25 elicits a multipotent progenitor cell population that promotes T(H)2
667 cytokine responses. *Nature*. **464** (7293), 1362-1366 (2010).

668 28 Hoyler, T. et al. The transcription factor GATA-3 controls cell fate and maintenance of type
669 2 innate lymphoid cells. *Immunity*. **37** (4), 634-648 (2012).

670 29 Nakajima, H., Shores, E. W., Noguchi, M., Leonard, W. J. The common cytokine receptor
671 gamma chain plays an essential role in regulating lymphoid homeostasis. *The Journal of*
672 *Experimental Medicine*. **185** (2), 189-195 (1997).

673 30 Warren, K. J. et al. RSV-specific anti-viral immunity is disrupted by chronic ethanol
674 consumption. *Alcohol*. <http://dx.doi.org/10.1016/j.alcohol.2016.08.001> (2016).

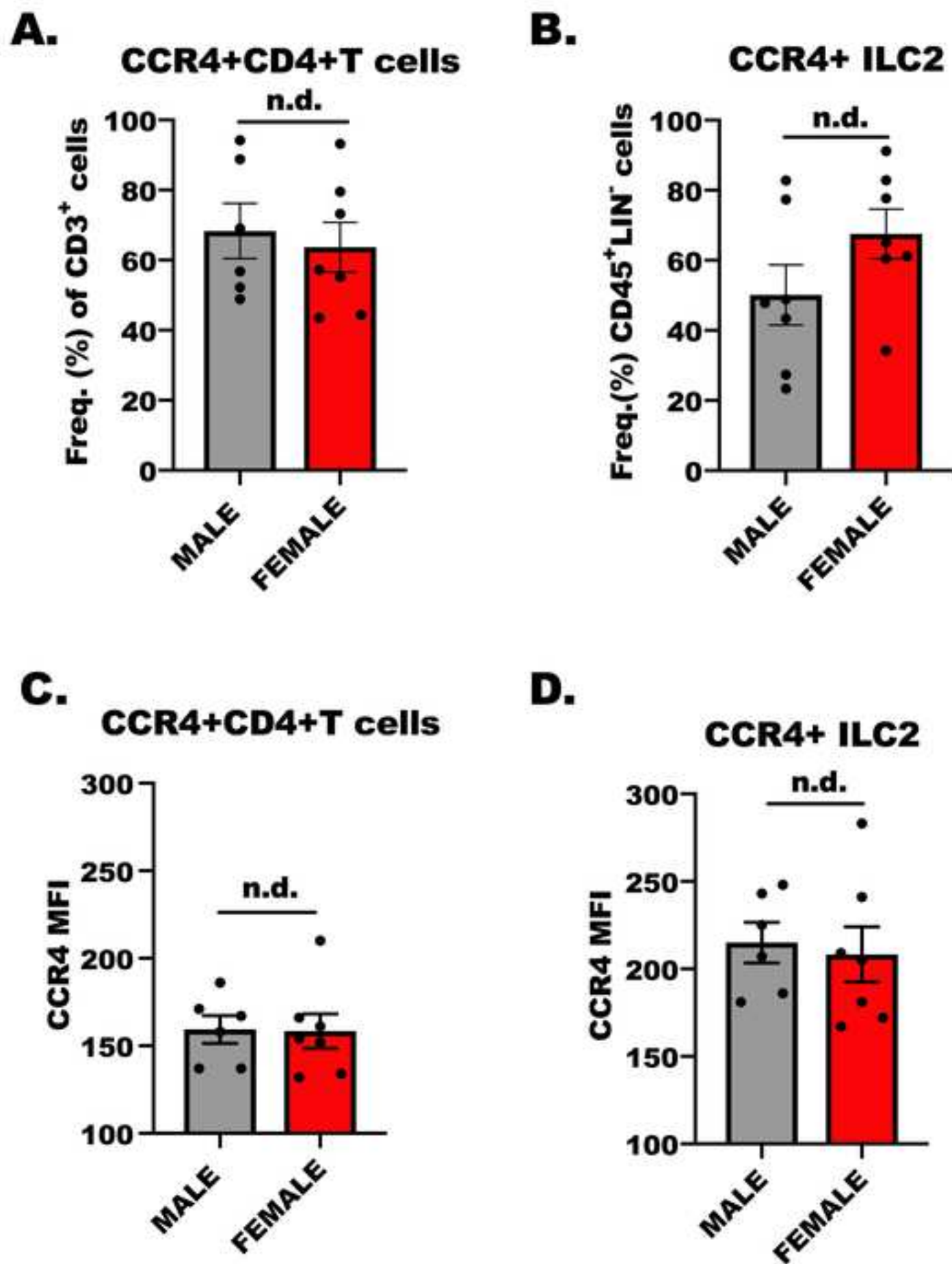
675 31 Warren, K. J., Poole, J. A., Sweeter, J. M., DeVasure, J. M., Wyatt, T. A. An association
676 between MMP-9 and impaired T cell migration in ethanol-fed BALB/c mice infected with
677 Respiratory Syncytial Virus-2A. *Alcohol*. [10.1016/j.alcohol.2018.09.009](https://doi.org/10.1016/j.alcohol.2018.09.009) (2018).

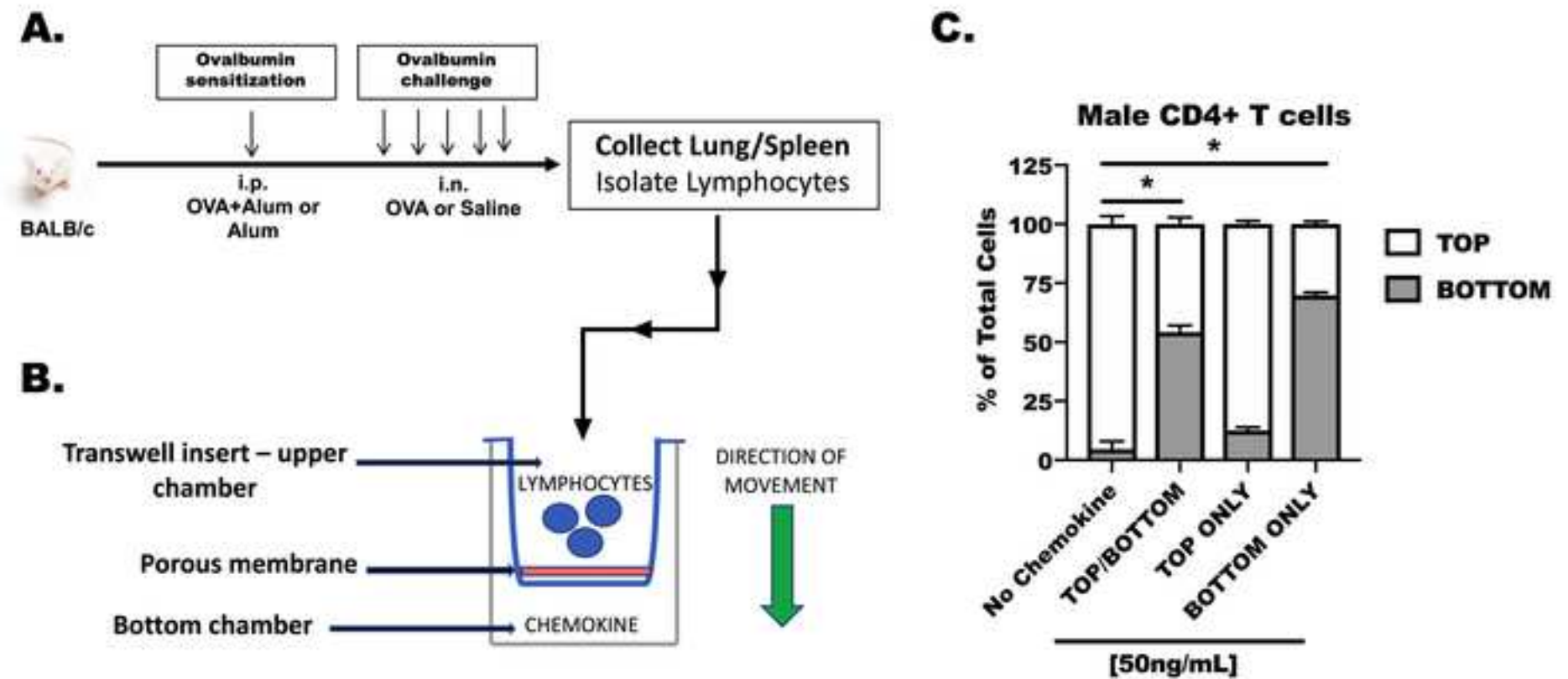
678 32 Molteni, R. et al. A novel device to concurrently assess leukocyte extravasation and
679 interstitial migration within a defined 3D environment. *Lab on a Chip*. **15** (1), 195-207
680 (2015).

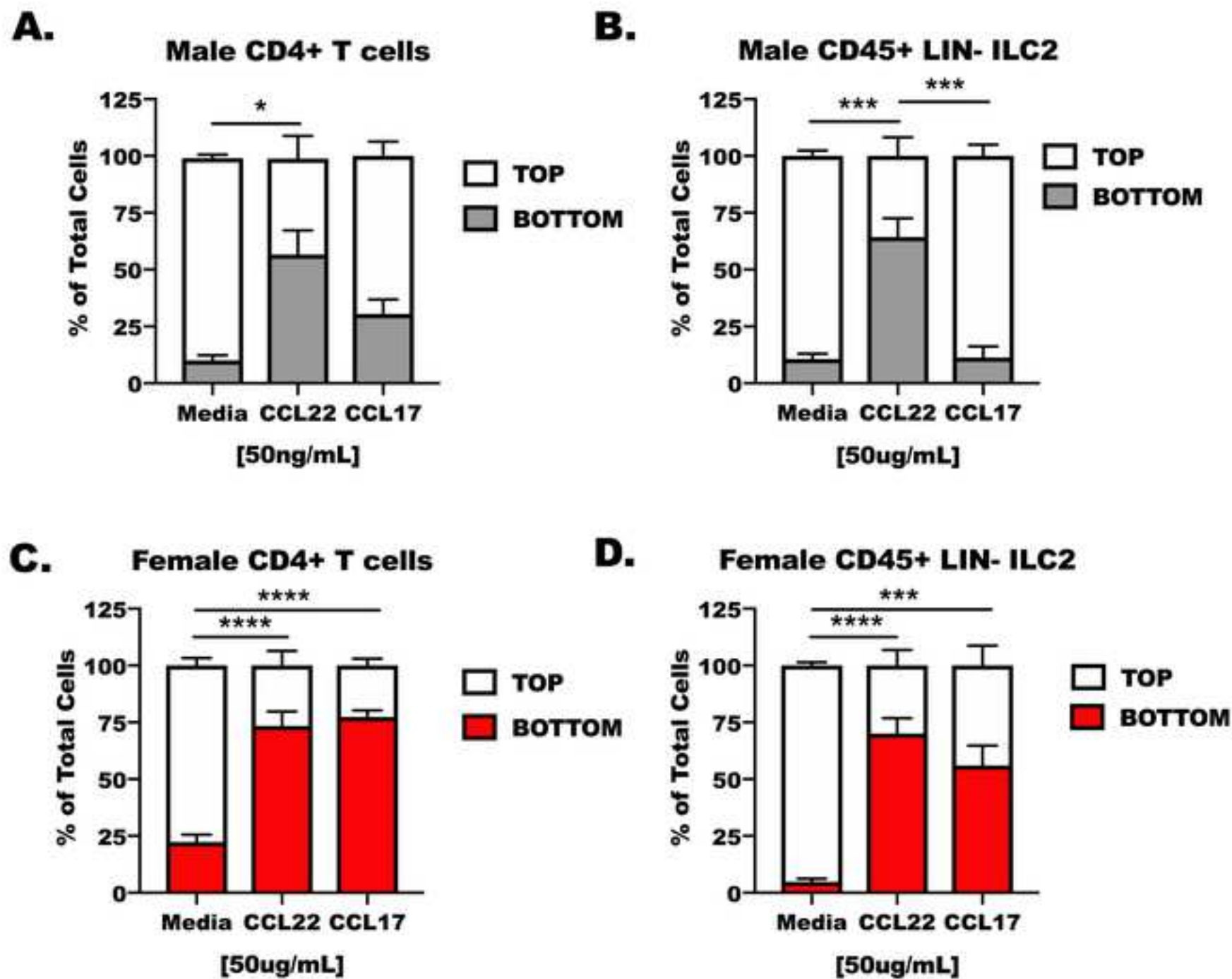
681 33 Bersini, S. et al. Human in vitro 3D co-culture model to engineer vascularized bone-
682 mimicking tissues combining computational tools and statistical experimental approach.
683 *Biomaterials*. **76**, 157-172 (2016).

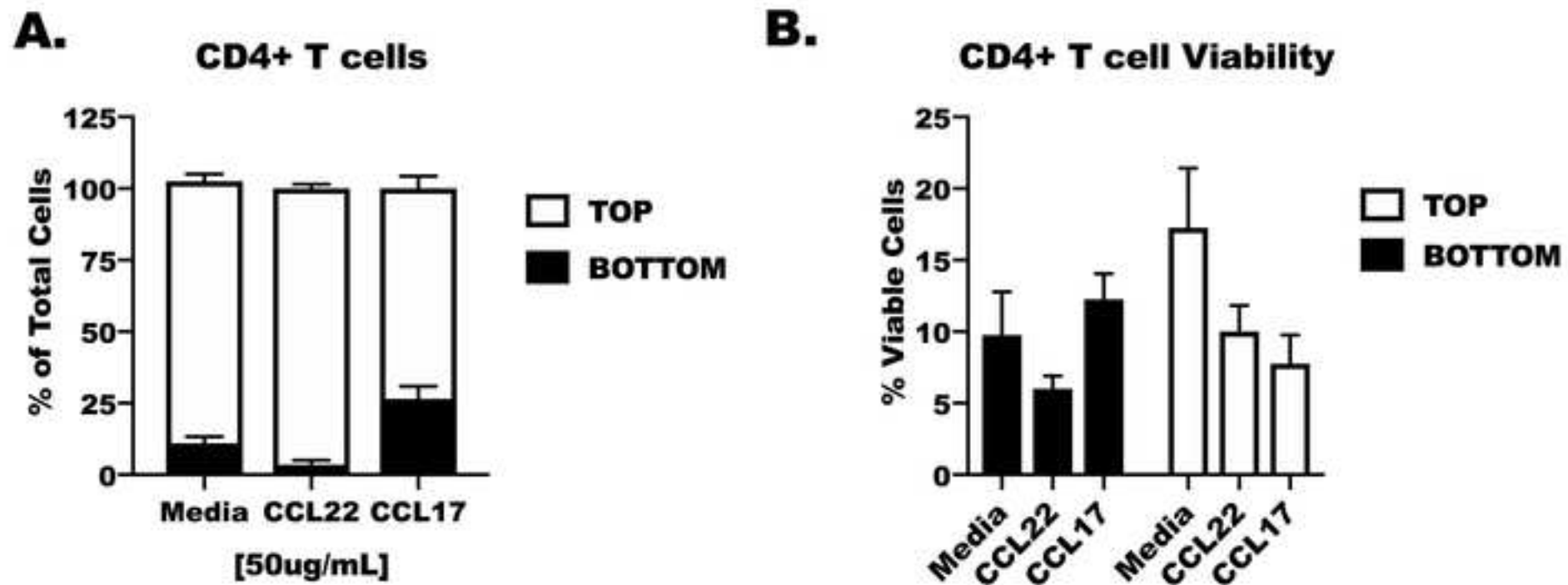
684 34 Jeon, J. S. et al. Human 3D vascularized organotypic microfluidic assays to study breast
685 cancer cell extravasation. *Proceedings of the National Academy of Sciences of the United*
686 *States of America*. **112** (1), 214-219 (2015).

687









Name of Material/ Equipment	Company
0.4% Trypan Blue	Sigma-Aldrich
1 mL syringe	BD Bioscience
100x Penicillin-Streptomycin, L-Glutamine	Gibco
15 mL conical tubes	Olympus Plastics
3 um transwell inserts	Genesee Scientific
3x stabilizing fixative	BD Pharmigen
5 mL polystyrene tubes	STEM Cell Technologies
50 mL conical tubes	Olympus Plastics
8-chamber easy separation magnet	STEM Cell Technologies
ACK Lysing Buffer	Life Technologies Corporation
Advanced cell strainer, 40 um	Genesee Scientific
Aluminum Hydroxide, Reagent Grade	Sigma-Aldrich
anti- mouse CCR4; APC-conjugated	Biolegend
anti-mouse CD11b	BD Pharmigen
anti-mouse CD11c; PE eFluor 610	Thermo-Fischer Scientific
anti-mouse CD16/32, Fc block	BD Pharmigen
anti-mouse CD19; APC-eFluor 780 conjugated	Thermo-Fischer Scientific
anti-mouse CD3; PE Cy 7-conjugated	BD Pharmigen
anti-mouse CD45; PE conjugated	BD Pharmigen
anti-mouse ICOS (CD278)	BD Pharmigen
anti-mouse NK1.1 (CD161); FITC-conjugated	BD Pharmigen
anti-mouse ST2 (IL-33R); PerCP Cy5.5 conjugated	Biolegend
Automated Cell Counter	BIORAD
Automated Dissociator	MACS Miltenyi Biotec
Bovine Serum Albumin, Lyophilized Powder	Sigma-Aldrich
Cell Counter Clides	BIORAD
Chicken Egg Ovalbumin, Grade V	Sigma-Aldrich
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Dissociation Tubes	MACS Miltenyi Biotec
FACS Buffer	BD Pharmigen
Heat Inactivated-FBS	Genesee Scientific
mouse CCL17	GenScript
mouse CCL22	GenScript
mouse CD4+ T cell enrichment kit	STEM Cell Technologies
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Comments/Description

U-100 Syringes Micro-Fine 28G 1/2" 1cc

Dilute to 1x in RPMI media

polypropylene tubes

24-well plate containing 12 transwell inserts

Prepare 1x solution according to manufacturers protocol

polypropylene tubes

nylon mesh, 40 micron strainers

20 mg/mL

0.5 ug/test

0.5 ug/test

0.25 ug/test

0.5 ug/test

0.5 ug/test

0.25 ug/test

0.5 ug/test

0.5 ug/test

0.25 ug/test

0.5 ug/test

0.5% in serum-free RPMI

500 ug/mL

25 U/mL in RPMI

1 drop per control tube

1x PBS + 2% FBS, w/ sodium azide; stored at 4 °C

10% in complete RPMI & ILC2 Expansion Media

50 ng/mL

50 ng/mL

20 ng/mL

20 ng/mL

1 X PBS + 2% FBS; stored at 4C

0.9% Sodium Chloride



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Date:

3/28/19

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Reply-To: Phillip Steindel <phillip.steindel@jove.com>

Date: Wednesday, May 1, 2019 at 9:31 AM

To: Todd Wyatt <twyatt@unmc.edu>

Subject: Revisions required for your JoVE submission JoVE60060 - [EMID:9cf23e8a325605f4]

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Dear Dr. Warren,

Your manuscript, JoVE60060 "Assessment of lymphocyte migration in an ex vivo transmigration system," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **June 5, 2019**.

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Best,

Phillip Steindel, Ph.D.

Review Editor

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Editorial comments:

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2. Please ensure that the manuscript is formatted according to JoVE guidelines—letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps. [This is done.](#)
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Specific Protocol steps:

1. Please provide more details (or a reference) on lung and spleen excision. [A reference has been added to the text of the manuscript.](#)

Figures:

1. Please explain the error bars in the figure legends. [The error bars have been explained in the figure legends now.](#)

References:

1. Please do not abbreviate journal titles.

[This was an oversight in the original submission. All the titles have been corrected.](#)

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol. [We have double checked the Materials and Equipment list, everything should now be included in this document.](#)

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a methodology for lymphocyte migration towards a chemoattractant using a modified version of the traditional Boyden chamber.

Protocol described in the manuscript is simple but not necessarily cost effective as claimed by the authors.

In light of many similar protocols available in the literature, it is unclear if this manuscript in the present form adds up to existing literature.

Major Concerns:

Lymphocytes can migrate through pores without the presence of chemokines. I have never seen passive migration of T cells through 3 micron-sized pores. The presence of chemokine is absolutely necessary for the migration to occur. At the smallest size a T cell may be 7 micron across, unless they are dividing/proliferating at which stage they get larger (7-20 microns), as such we took this reviewer's suggestion just below regarding placing chemokine in the top and bottom chambers and show in these new results that migration in this transwell system is an active process that requires chemokine (see newly added Figure 2)

Incubation period of 72h is long enough for all the lymphocytes to migrate. This is a typo in the original submission, the T cells and ILC2 were incubated from 48 hours total. In addition, we do not see passive migration of lymphocytes in these experiments.

In order to conclusively show that lymphocytes are dependent on chemokines one needs to use pertussis toxin to block the migration. To confirm the dependence of CD4+ T cell migration we repeated and experiment using the suggestion in the very next comment to confirm that this is a chemokine-dependent process. These new data are contained in Figure 2.

If chemokines are used in the top chamber, do the lymphocytes stop migrating? Yes, the CD4+ T cells do not migrate when there is chemokine in the top chamber. This was an excellent suggestion by the reviewer. Please see new Figure 2.

Rationale of performing suboptimal migration experiment is not well explained in the text. Generating suboptimal results is required by the journal to help someone performing the experiment know what to expect when the experiment doesn't work properly. We have added a sentence to provide a rationale for producing these results.

Minor Concerns:

In the method section following clarifications are needed,

1. Is the FBS heat inactivated or complement deactivated? It is now clearly stated that the FBS was heat-inactivated.
2. Please clarify if the nature of 40uM cell strainer. Is this a nylon strainer? Yes, you are correct the strainer was nylon, and we have added this detail to the manuscript as well.
3. Fig 2B shows that CCL17 migration (bottom chamber) is significant (as denoted by asterisks) but the text states otherwise. Please correct the discrepancy. We have corrected this in the manuscript.
4. Fig. 2A if CD4 cells migrate from top to bottom chamber, then cells in the top chamber need to go down. Based on the bar graph there is no change in number of cells in the top chamber. We have reviewed this figure, and the purpose of the graph is to compare the media, CCL22 and CCL17 bars. When comparing each bar the percentage of CD4+ T cells do go down in the top chamber when comparing media to CCL22 treatments.

Reviewer #2:

Manuscript Summary:

I am not an expert of biological procedures to obtain cells ex vivo so I am not entitled to check the single laboratory steps for each procedure. Anyway the description is very detailed and complete. Each step is logically well defined.

In the experiments, population of cells (CD4 T and ILC2) from mice (placed separately in the top chambers of a transmigration system) are induced to transmigrate across a porous membrane by the presence of chemokines in the bottom chambers of a transmigration system.

The proposed experiments are very interesting because they allow the observation of a complex phenomenon in a simple and quite cheap set up. The Boyden chamber set up is, in literature, a very well known set

up, many times described in literature for chemotactic investigations.

Together with these advantages, the Boyden chamber set up has been recognized as a set up with many limits that keep the experimental conditions very far from the "real" ones. These limits are mostly related to the mechanobiology: the mechanical stimuli from the blood and from the tissues on cells are absent.

As the authors said, these experiments is very useful to determine the reaction or not of a cell population to a given chemokine.

The authors show also some results able to validate the results of the main experiment : in the wrong conditions of %CO₂ and temperature, survival cells do not transmigrate. In the absence of chemical stimulus the transmigration happens but it is a very negligible contribution. These results show that the experiment "sees" the effect of the chemokine on the transmigration of cells through the transwell insert.

[Thank you for these comments.](#)

Major Concerns:

In the description of the framework: on one side the Boyden chamber (easy going and cheap), on the other side the observation in vivo (expensive, complex, cruel .. etc) there are some technical solutions in the middle that you may consider to talk about.

These technical solutions are microfluidics lab on a chip, where researchers try to mimic in vitro the "real" environment, by introducing mechanical and fluid dynamic stimuli to complete the chemotactic condition, described by the Boyden chamber approach. [We appreciate these suggestions and have added some discussion about alternatives or other systems that lie in 'the middle' for the readers to consider.](#)

Here some examples:

Lab Chip. 2015 Jan 7;15(1):195-207. doi: 10.1039/c4lc00741g. A novel device to concurrently assess leukocyte extravasation and interstitial migration within a defined 3D environment. Molteni R, Bianchi E, Patete P, Fabbri M, Baroni G, Dubini G, Pardi R.

Proc Natl Acad Sci U S A. 2015 Jan 6;112(1):214-9. doi: 10.1073/pnas.1417115112. Epub 2014 Dec 18. Human 3D vascularized organotypic microfluidic assays to study breast cancer cell extravasation. Jeon

JS, Bersini S, Gilardi M, Dubini G, Charest JL, Moretti M, Kamm RD.

You may consider to insert some sentences about these kind of devices/approach. [Thank you to this reviewer for including these references and insight into ways to improve the manuscript. Again, we have expanded this discussion in the manuscript.](#)

Although a complete description of the set up is not available in this document (multiwells + transwell inserts but anything else but the pore size.. the thickness of the membrane, i.e.), the use of a Boyden chamber kind of set up makes the experiment more assimilable to an In vitro experiment than to an ex Vivo. In my opinion you should consider to review the title. [The reason we used ex vivo versus in vitro in the title is because the use of allergen-challenged animals is absolutely necessary for the success of these experiments. In allergen-challenged animals an OVA-specific T cell response develops in vivo where T cells and ILC2 within the animals specifically upregulated CCR4, which are specific for CCL17 and CCL22. One of the novel components of this manuscript is the assessment of the ILC2 movement towards these chemokines as well. These ILC2 need to be harvested from allergen-challenged animals as well in order to get enough of this rare cell to carry out the transmigration assay. As such, there is an ex vivo component to the work because the T cells/ILC2 are grown in vivo under conditions that make them well suited for this specific chemokinetic assay. In vitro would apply perhaps if the animals were](#)

naïve OR if the cells were from an immortalized cell line, e.g. Jurkat T cells.

Together with the video, a schematic representation of the set up would help the reader (and the spectator of the video!) to understand how the system works, direction of transmigration, chemotactic gradients and characteristic dimensions of the phenomena. [This is a good point by the reviewer. We have developed a schematic to aid the reader of the manuscript and those watching the video to understand the movement of the cells and important characteristics of the assay.](#)

In the list of the materials/equipment I suppose that the 24-multiwell is missing (should be there? I may put it because it is the main piece of the set up, together with the inserts). [Yes, the inserts come shipped in a 24-well plate, however, this is a great suggestion and this piece of the description that should be provided. This has now been added to the Materials/Equipment and in the manuscript it has been clarified.](#)

Please check Typos in the titles of fig.1, section D -> + [This has been corrected in Figure 1, and the remaining Figures were checked for consistency.](#)

Please check fig 2, titles of sec B and C. CD45 ? check the legend [We removed CD45 from the titles. Both CD4+ T cells and ILC2 are both CD45+ \(pan lymphocyte marker\). This details is unnecessary as it is included in the text of the manuscript.](#)

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