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TITLE:

Imaging of In Situ Interferon Gamma Production in the Mouse Spleen following *Listeria monocytogenes* Infection

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SUMMARY:

Here, we describe a simple confocal imaging method to visualize the in situ localization of cells secreting the cytokine Interferon gamma in murine secondary lymphoid organs. This protocol can be extended for the visualization of other cytokines in diverse tissues.

ABSTRACT:

Cytokines are small proteins secreted by cells, mediating cell-cell communications that are crucial for effective immune responses. One characteristic of cytokines is their pleiotropism, as they are produced by and can affect a multitude of cell types. As such, it is important to understand not only which cells are producing cytokines, but also in which environment they do so, in order to define more specific therapeutics. Here, we describe a method to visualize cytokine production in situ following bacterial infection. This technique relies on imaging cytokine-producing cells in their native environment by confocal microscopy. To do so, tissue sections are stained for markers of multiple cell types together with a cytokine stain. Key to this method, cytokine secretion is blocked directly in vivo before harvesting the tissue of interest, allowing for detection of the cytokine that accumulated inside the producing cells. The advantages of this method are multiple. First, the microenvironment in which cytokines are produced is preserved, which could ultimately inform on the signals required for cytokine production and the cells affected by those cytokines. In addition, this method gives an indication of the location of the cytokine production in vivo, as it does not rely on artificial in vitro re-stimulation of the producing cells. However, it is not possible to simultaneously analyze cytokine downstream signaling in cells that receive the cytokine. Similarly, the cytokine signals observed corresponds only to the time-window during which cytokine secretion was blocked. While we describe the visualization of the cytokine Interferon (IFN) gamma in the spleen following mouse infection by the intracellular bacteria *Listeria monocytogenes*, this method could potentially be adapted to the visualization of any

cytokine in most organs.

INTRODUCTION:

Orchestrating an efficient immune response against a pathogen requires complex integration of signals displayed by a variety of immune cells that are often dispersed among the organism. In order to communicate, these cells produce small soluble proteins with multiple biological functions that act as immunomodulators named cytokines. Cytokines control cell recruitment, activation and proliferation and hence are known to be key players in the promotion of immune responses¹. Effective immune responses require cytokines to be released in a very organized spatiotemporal pattern connecting specific cells to induce specific signals. Therefore, it is crucial to study cytokine production and its signaling in situ, taking into account the microenvironment in which cytokines are produced.

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive intracellular bacterium used as a prime model to study immune responses to intracellular pathogens in mice. One cytokine, IFN gamma (IFN γ) is produced rapidly, within 24 h following *L. monocytogenes* infection. It is necessary for pathogen clearance, as mice knocked out for IFN γ are highly susceptible to *L. monocytogenes* infection². IFN γ is pleiotropic and produced by multiple cells following infection³. While IFN γ produced by natural killer (NK) cells is required for direct anti-bacterial activity⁴, IFN γ from other sources have been shown to have other functions. Indeed, we and others recently found that IFN γ produced by CD8⁺ T cells has a specific function in directly regulating T cell differentiation⁵⁻⁷. As such, understanding which cells produce IFN γ (and in which microenvironment) is crucial to dissect its function.

The most common technique to study cytokine production relies on intracellular cytokine staining analyzed by flow cytometry. This method allows the simultaneous detection of multiple cytokines combined with cell surface markers within a single sample, providing an extremely useful tool to study cytokine production. However, using the aforementioned technique implies losing any spatial information. In addition, cytokine detection often relies on in vitro re-stimulation to enable cytokine detection. As such, the capacity of a given cell to produce a cytokine is analyzed, and it does not necessarily correlate with actual cytokine secretion in situ. Other methods use reporter mice for which fluorescent protein expression correlates with cytokine transcription and allows for visualization on a single-cell level⁸. Although this method can track cytokine transcription in situ, there are a limited number of cytokine-reporter mice available. In addition, transcription, translation and secretion can sometimes be unlinked, and fluorescent proteins have a different half-life than the cytokine they report, making this method sometimes not adequate for in situ cytokine visualization.

Here, we describe a method to visualize in situ cytokine production by confocal microscopy at single cell resolution. This technique enables the visualization of the cellular source and surrounding niche within the tissue. This protocol specifically describes the visualization of IFN γ production in the spleen of *L. monocytogenes* infected mice, focusing here on IFN γ production by NK cells and antigen specific CD8⁺ T cells. However, it can be extended and adapted to the characterization of any cytokine production in the context of other situations where cytokines

are produced such as infection, inflammation or autoimmune diseases, as long the targeted cytokine can be retained in cells by intracellular protein transport inhibitor.

PROTOCOL:

All experiments involving mice were in agreement with the UK Scientific Procedures Act of 1986.

1. Adoptive transfer of CD8+ T cells in mice

1.1. Isolate ovalbumin (OVA)-specific CD8+ T cells (OTI) expressing green fluorescent protein (OTI-GFP) or red fluorescent protein (OTI-RFP) from lymph node suspension of T-cell receptor transgenic mice^{9,10} using a mouse CD8+ T cell isolation kit as per manufacture instructions. Prepare the cell suspension by smashing lymph nodes using a syringe plunger, as previously described¹¹.

1.2. Transfer OTI-GFP or OTI-RFP cells (3×10^6 cells) into C57BL/6 wild type mice recipients by intravenous injection as described by Cahalan, et al.¹². Use mice that are typically 6–12 week of age.

NOTE: This step is optional and only required for tracking antigen-specific CD8+ T cells.

2. *Listeria monocytogenes* infection

2.1. Expand *L. monocytogenes* genetically modified to express OVA (LM-OVA)¹³ to an exponential phase of growth in broth heart infusion at 37 °C under gentle agitation until the OD₆₀₀ reaches 0.08–0.1, as previously described¹⁴.

2.2. Inject 100 µL (maximum volume = 200 µL) of 0.1–0.5 LD₅₀ LM-OVA diluted in phosphate-buffered saline (PBS) by intravenous injection using a 29 G insulin syringe, into C57BL/6 wild type mice recipients bearing OTI-GFP or OTI-RFP cells when indicated.

NOTE: In our hands, 0.1 x LD₅₀ LM-OVA corresponds to 2×10^4 colony forming units (CFU). *L. monocytogenes* genetically modified to express OVA is used to activate the previously transferred OTI CD8+ T cells, but other strains of *L. monocytogenes* can be used.

3. Treatment with Brefeldin A (BFA) to block cytokine secretion

3.1. Inject 250 µg of BFA in 200 µL of PBS intraperitoneally 6 h before mouse sacrifice using a 29 G insulin syringe.

NOTE: Lyophilized BFA is first resuspended in dimethyl sulfoxide (DMSO) to prepare stock of 25 mg/mL concentration. The BFA is then diluted in PBS at room temperature (RT) to avoid crystallization prior to injection. Inhibition of cytokine secretion induces accumulation of IFN γ in cells. This is crucial for cytokine detection.

4. Harvesting the spleen

4.1. Euthanize the mice using rising concentration of CO₂ followed by cervical dislocation.

NOTE: Follow the local institution guidelines for humane euthanasia of mice.

4.2. Cleanse the abdomen with 70% ethanol, make an incision with scissors to make a 1–2 cm cut through the skin on the left flank of the mouse, where the spleen is located. Carefully make an incision in the peritoneum to expose the spleen and take it out with tweezers. Harvest the spleen, being careful not to squeeze it with forceps or cut it to avoid disrupting the spleen architecture.

5. Fixation of the spleen with paraformaldehyde (PFA)

5.1. Prepare the fixative solution by mixing 3.75 mL of PBS and 3.75 mL of 0.2 M L-lysine. Add 21 mg of sodium m-periodate and mix well. Then add 2.5 mL of 4% PFA and 20 µL of 12 N NaOH.

NOTE: Use the fixative solution on the same day and discard the excess. Do not store it. This fixation step is important if the sample contains fluorescent proteins such as GFP. Do not use PFA containing traces of methanol, as it denatures fluorescent proteins.

CAUTION: PFA is toxic and must be handled with caution.

5.2. Submerge the spleen in the fixative and fix for a minimum of 4 h, typically 16–20 h at 4°C under gentle agitation.

5.3. Discard the fixative solution and add 5 mL of PBS for 5 min at RT under gentle agitation.

5.4. Replace the PBS with 5 mL of fresh PBS incubate for 1 hour at 4 °C under gentle agitation.

5.5. Replace the PBS with 5 mL of 30% sucrose, incubate for 12–24 h.

NOTE: This method helps maintain the tissue morphology. After incubation with sucrose solution, the organ should sink at the bottom of the plate.

6. Freezing and sectioning

6.1. Put dry ice in a large receptacle and place a smaller receptacle inside containing around 50 mL of pure methanol and a few pieces of dry ice.

6.2. Gently dry the spleen on a lint-free wipe.

6.3. Place the spleen inside a base mold containing a drop of optimum cutting temperature (OCT) compound at the bottom. Be careful not to produce any bubbles. Add OCT over the spleen.

177 6.4. With forceps, deposit the base mold on the surface of the methanol, making sure that it does
178 not touch the OCT. Freezing must take less than 30 s.

179
180 6.5. When it is frozen, proceed with sectioning.

181
182 NOTE: Frozen spleen can be kept at -80 °C for several months.

183
184 6.6. Section the tissue using a cryomicrotome.

185
186 6.6.1. Set the chamber temperature on the cryostat should to -21 °C. Cut sections of the desired
187 thickness (usually around 10 µm). This protocol works with thicknesses up to 30 µm.

188
189 6.7. Collect sections onto glass microscope slides (see the **Table of Materials**) and inspect visually.

190
191 NOTE: Sections can be kept at -80°C for several months.

192 193 7. Immunofluorescent staining

194
195 7.1. Allow the section to come to RT.

196
197 7.2. Draw a circle with a liquid blocker (e.g., PAP pen) around the tissue section. Draw outside
198 the OCT or it will not stick.

199
200 7.3. Once it has dried, rehydrate the sample by placing PBS on the tissue section for 5 min.

201
202 NOTE: The volume put on the section depends on the size of the section. We typically use 100–
203 300 µL. Do not let the sections dry once they are rehydrated.

204
205 7.4. Rinse with PBS at least twice to ensure that the section is well adhered to the slide.

206
207 7.5. Add blocking solution to the section to decrease non-specific binding of antibodies.

208
209 7.5.1. Prepare blocking solution as follow: PBS with 2% fetal calf serum (FCS), 2.5 µg/mL Fc
210 receptor blocker (anti-mouse CD16/32). Then add 2–5% normal serum of the species of each
211 secondary antibody of the staining panel.

212
213 NOTE: If the antibodies are directly conjugated/biotinylated, add 5% normal serum of the species
214 of each primary antibody. If a primary antibody and one of the secondary antibodies are from
215 the same species (e.g., primary antibody raised in rabbit and secondary rabbit anti-rat), do not
216 use the normal serum species as it will increase the background signal.

217
218 7.5.2. Gently remove the PBS from the section by aspiration and add 100 µL of the blocking
219 solution per sample section. Incubate in a covered wet chamber for a minimum of 1 h at RT.

220

7.6. Stain with primary antibodies.

7.6.1. Dilute the primary antibodies at the optimal concentration in the blocking solution. The general starting point antibody concentration is 5 µg/mL, but it should be optimized for each antibody and tissue.

NOTE: If the antibodies are directly conjugated, centrifuge the antibody mix at 17.135 x g (13.500 rpm) for 15 min at 4 °C before using it. Fluorophores can precipitate. This step will pellet the precipitates and thereby prevent non-specific deposition of the precipitated antibodies on the slide.

7.6.2. Replace the blocking solution with the primary antibody mix for each sample.

7.6.3. Incubate for 4 h at RT or overnight (OVN) at 4 °C in a covered wet chamber.

7.7. Perform washing.

7.7.1. Prepare Wash Buffer by adding 2% FCS to PBS.

7.7.2. Wash 4 times with wash buffer: one quick (without incubation), one for 10 min and two for 5 min.

7.8. Staining with secondary antibodies.

7.8.1. Dilute the secondary antibodies of interest at the optimal concentration in the blocking solution. Centrifuge the mix, as described for the primary antibodies.

7.8.2. Remove the final wash solution. Add the secondary antibody mix on top of the section and incubate for 1–4 h at RT in a covered wet chamber.

7.9. Wash 4 times with wash buffer: one quick (without incubation), one for 10 min and two for 5 min.

7.10. Remove the final wash solution. Allow the PBS to evaporate but do not over-dry the section. Place a drop of the mounting medium on top of the sample and carefully place the cover glass on top of it. The mounting medium must recover the whole section. Let it polymerase OVN at RT protected from light.

NOTE: Draw a circle around the section on the reverse side of the slide before applying the mounting medium. Once the mounting medium is applied, the tissue might become difficult to see.

7.11. Store the slides in the dark at 4 °C until ready to image.

8. Imaging and analysis

8.1. Perform imaging of the staining with a confocal microscope.

NOTE: In this protocol, an inverted spectral laser scanning microscope was used (see the **Table of Materials**), together with the objectives 10x/NA 0.40 or 60x/NA 1.4 (for analysis of cytokine sub-cellular localization). Wavelengths of excitation and emission are displayed for each fluorophore and fluorescent protein in the **Table of Materials**.

8.2. Perform analysis and quantification as required using image processing software (e.g., Imaris or Fiji).

REPRESENTATIVE RESULTS:

IFN γ produced within the first 24 h after *Listeria monocytogenes* infection is critical to control the spread of this pathogen. Using this protocol, we can visualize not only which cells are producing IFN γ but also whether they are located in a specific microenvironment. To help us delineate the architecture of the spleen, we labeled cells known to have particular location within the spleen. The marker F4/80 labels all macrophages and highlights the red pulp. The marker B220 labels B cells and highlights B cell follicles surrounding the T cell zone. The marker CD169 labels marginal zone macrophages, surrounding the white pulp (**Figure 1**). Most OTI cells, whether they express IFN γ or not, are present in the white pulp and as such, all images are those of the white pulp, unless indicated.

One critical step in this protocol is the use of BFA to inhibit cytokine secretion. Indeed, the detection of IFN γ by NK cells was greatly impaired when mice were not treated with BFA (**Figure 2**). Using our protocol, we could find that at least two cell types produce IFN γ 24 h after infection—NK cells and antigen-specific CD8 $^{+}$ T cells (**Figure 3**)—similarly to what has been found previously by flow cytometry³.

In situ imaging of IFN γ producing cells revealed that IFN γ production is not spread throughout the spleen, but concentrated into discreet areas (**Figure 4**). Indeed, we found that T cells were activated throughout the spleen (highlighted by T cell clustering), and this did not necessarily correlate with IFN γ production. One likely explanation is that IFN γ production is restricted to the location of the infected cells^{15,16}, and T cell activation—represented by clustering—can be supported by both infected (IFN γ positive) and non-infected (IFN γ negative) antigen-presenting cells. Other stains will be required to pinpoint the exact location and get an indication of the mechanism restricting IFN γ production to this area and its relationship to antigen transfer. Interestingly, we found that activated, clustered, antigen-specific T cells are located throughout the white pulp of the spleen but they produce IFN γ only in regions where NK cells are coexisting with them (**Figure 5**). As such, the presence of NK cells delineates a specific microenvironment in the white pulp, in which clustered T cells produce IFN γ as opposed to clustered T cells in the other part of the white pulp. This suggests that T cell activation is not sufficient to dictate IFN γ production at this time point.

Another interesting feature highlighted by our protocol is the different sub-cellular localization of IFN γ in NK versus CD8 $^{+}$ T cells⁵. As shown in **Figure 6**, while IFN γ localization in NK cells is diffused in the cytosol, CD8 $^{+}$ T cells often recruit IFN γ towards another T cell.

FIGURE LEGENDS:

Figure 1: Markers highlighting the spleen architecture. Mice were infected with 2×10^4 CFU LM-OVA and euthanized 24 h post infection. Spleen was explanted and processed as described in the protocol. **(A)** Sections were stained for NK cells (anti-NCR1 followed by anti-goat IgG-FITC; green), OTI-RFP cells (red) and macrophages (anti-F4/80-APC; magenta). RP = Red Pulp; WP = White Pulp. Scale bar represents 200 μ m. **(B)** Sections were stained for B cells (anti-B220-Pacific Blue; Blue), OTI-GFP cells (GFP signal shown in red) and marginal zone macrophages (anti-CD169-Alexa647; magenta). RP = Red Pulp; BF = B cell follicle; TZ = T cell zone. Scale bar represents 50 μ m. This is a representative image of 3 independent experiments (N = 4).

Figure 2: BFA treatment allows for the detection of intracellular IFN γ in situ. Mice were infected with 2×10^4 CFU LM-OVA and treated with BFA **(A)** or left untreated **(B)** after 18 h. Mice were euthanized 24 h post infection. Spleen was explanted and processed as described in the protocol. Sections were stained for NK cells (anti-NCR1 followed by anti-goat IgG-FITC; green), OTI-RFP cells (red) and IFN γ (anti-IFN γ -BV421; cyan). Scale bar represents 5 μ m. This is a representative image of NK cell-rich areas from 3 independent experiments (N = 3).

Figure 3: IFN γ producing cells in the spleen. Mice were infected with 2×10^4 CFU LM-OVA when indicated and treated with BFA after 18 h. Mice were euthanized 24 h post infection. Spleen was explanted and processed as described in the protocol. Sections were stained for NK cells (anti-NCR1 followed by anti-goat IgG-FITC; green), OTI-RFP cells (red) and IFN γ (anti-IFN γ -BV421; cyan). **(A)** Representative image of a spleen from an un-infected naïve mouse to demonstrate absence of IFN γ non-specific staining. White lines delineate the white pulp. WP = White pulp; RP = Red pulp. **(B)** Representative image of the white pulp from the spleen of a mouse infected by LM-OVA, showing invasion of NK cells to the white pulp and production of IFN γ by NK cells, OTI cells and non-labelled cells. Images are representative from 4 independent experiments (N = 4). Scale bars represent 70 μ m (A) and 20 μ m (B).

Figure 4: IFN γ production is restricted to specific areas in the spleen following LM-OVA infection. Mice were infected with 2×10^4 CFU LM-OVA and treated with BFA after 18 h. Mice were euthanized 24 h post infection. Spleen was explanted and processed as described in the protocol. All sections were stained for B cells (B220-Pacific Blue Ab, Blue) and IFN γ (anti-IFN γ -biotin followed by streptavidin-PE; cyan). OTI-GFP cells (GFP signal shown in red). Cyan lines correspond to areas of high IFN γ production. Those are representative images of 4 independent experiments (N = 4). **(A)** Sections were stained for marginal zone macrophages (anti-CD169-Alexa 647, magenta). Scale bar represents 50 μ m. **(B)** Sections were stained for all macrophages (F4/80). Scale bar represents 100 μ m.

Figure 5: IFN γ production by activated OTI cells occurs in a specific microenvironment. Mice

were infected with 2×10^4 CFU LM-OVA and treated with BFA after 18 h. Mice were euthanized 24 h post infection. Spleens were explanted and processed as described in the protocol. Sections were stained for NK cells (anti-NCR1 followed by anti-goat IgG-FITC; green), OTI-RFP cells (red) and IFN γ (anti-IFN γ -BV421; cyan). Green and red lines highlight NK and OTI cell zones, respectively. White arrow indicate examples of T cell clusters not producing IFN γ . Green arrows examples of T cell clusters producing IFN γ . Scale bar represents 100 μ m. This is a representative image of four independent experiments (N = 4).

Figure 6: Sub-cellular localization of IFN γ in NK cells and T cells. Mice were infected with 2×10^4 CFU LM-OVA and treated with BFA after 18 h. Mice were euthanized 24 h post infection. Spleen was explanted and processed as described in the protocol. All sections were stained for IFN γ (anti-IFN γ -BV421; cyan). White lines delineate cell edges and white arrows shows directionality of secretion. This is a representative image of two independent experiments (N = 5). **(A)**- OTI-RFP cells are shown in red. Scale bar: 5 μ m. **(B)** Sections were stained for NK cells (anti-NCR1 followed by anti-goat IgG-FITC; green. Scale bar: 2 μ m.

DISCUSSION:

In this manuscript, we present a method to visualize IFN γ production in the spleen following *L. monocytogenes* infection in mice. This protocol is simple and can be adapted to other tissues and cytokine triggers, but the following aspects have to be considered. Cells often rapidly secrete the cytokines they produce, and cytokines are rapidly picked up by neighboring cells. It is as such difficult to detect cytokines in situ. A common method to rapidly re-initiate cytokine production is to re-stimulate the cells ex vivo followed by cytokine detection in the media by enzyme-linked immunosorbent assay. In this context, any information about the spatial localization of the cytokine-producing cells is lost. In addition, cytokine production following re-stimulation does not necessarily reflect whether cytokines are actually produced and secreted in vivo, but rather indicates the capacity of a given cell population to produce cytokines. Therefore, both methods will provide different information and one should consider which information is most valuable for their experiment.

In order to detect intracellular cytokines, our method uses an intracellular protein transport inhibitor to trap cytokines inside cells and increase signal detection. However, it is important to note that these inhibitors affect the normal transportation of proteins from the endoplasmic reticulum (RE) to the Golgi apparatus and to the secretory vesicle impairing their release, which could cause toxicity. As a consequence, BFA, or other inhibitor, should be used for a short period of time, typically no more than a few hours. Hence, it is important to find the right balance between the inhibitor dose and time of treatment in order to optimize the level of cytokines trapped inside the cell without causing serious cytotoxic effects. These variables can differ between cytokines and the route of administration for the BFA. In our infection model, the BFA was administrated intraperitoneally in order to provide a rapid systemic dispersion, but it can also be delivered intravenously.

The most commonly used intracellular protein transport inhibitors are BFA, used here, and monensin (MN). These inhibitors are often used indistinctly to accumulate and study cytokine

production but they have slight differences in their mechanisms of action. MN inhibits transportation of proteins within the Golgi apparatus hence accumulating proteins in the Golgi¹⁷ while BFA prevents coatamer protein complex-I recruitment inhibiting the retrograde movement of proteins to the endoplasmic reticulum (ER) promoting accumulation of cytokines in the ER¹⁸. As such, choosing the best intracellular protein transport inhibitor will depend on different factors, such as the cytokine to be detected. For example, it has been shown in lipopolysaccharide-induced intracellular staining of monocytes that BFA is more efficient to measure the cytokines IL-1 β , IL-6 and TNF than MN¹⁹.

This protocol involves the visualization of the cytokine in situ by confocal microscopy and therefore there are only a limited number of markers than can be used to study the cytokine-producing cells and their microenvironment. It is also necessary to consider that protein transport inhibitors such as BFA or MN disturb the normal expression of several proteins and hence their use when studying the simultaneous expression of certain activation cell surface markers has to be approached carefully. For example, BFA but not MN blocks the expression of CD69 in murine lymphocytes²⁰. Despite this limitation, confocal imaging enables sub-cellular localization of cytokines, as well as the direction of the cytokine secretion within the cell. The data generated using this protocol suggest that NK cells tend to secrete IFN- γ in a diffuse pattern while CD8+ T cells seem to direct the IFN γ secretion towards other CD8+ T cells that are in direct interaction with them⁵.

To conclude, this protocol is suitable to visualize a variety of cytokines in situ and identify producing cells and their microenvironment following many triggers such as infection or autoimmunity. The information obtained is instrumental to understand the importance of the in vivo spatial orchestration of different cell types and the cytokine they produce, necessary for an efficient immune response.

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DISCLOSURES:

The authors have nothing to disclose.

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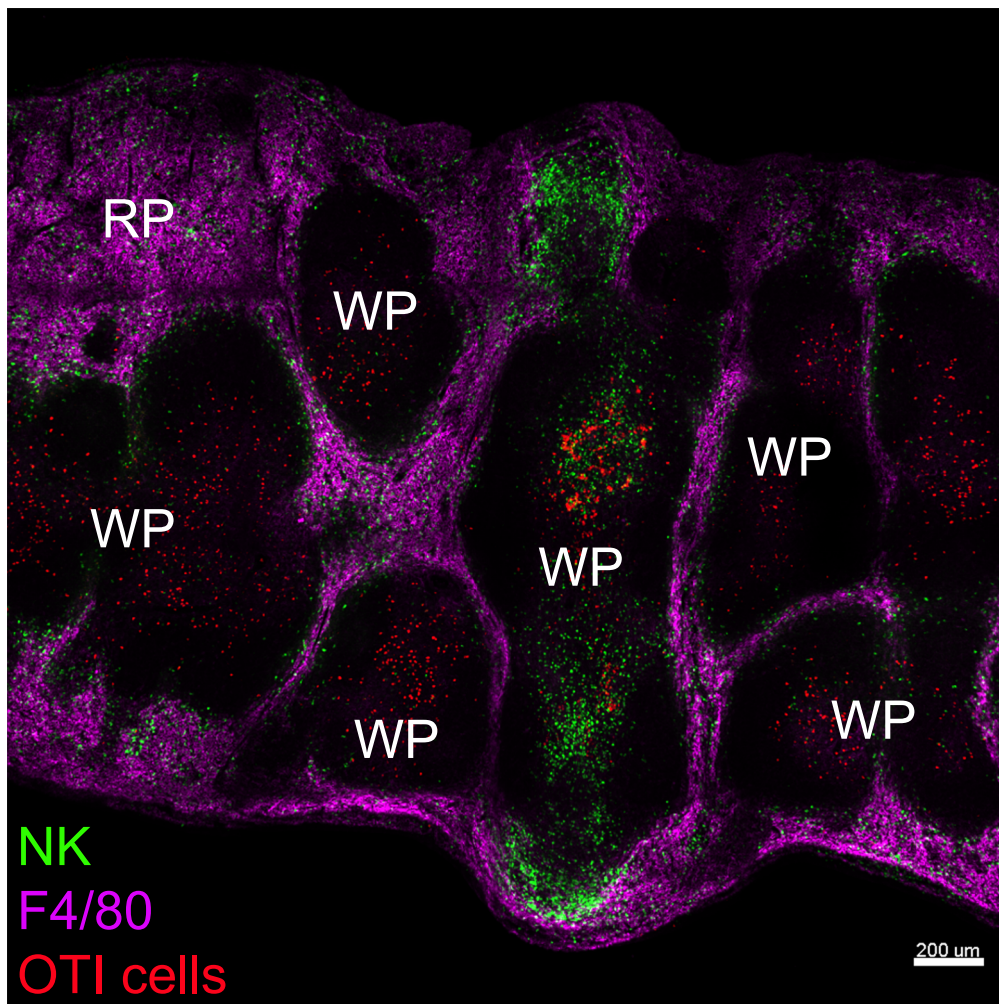
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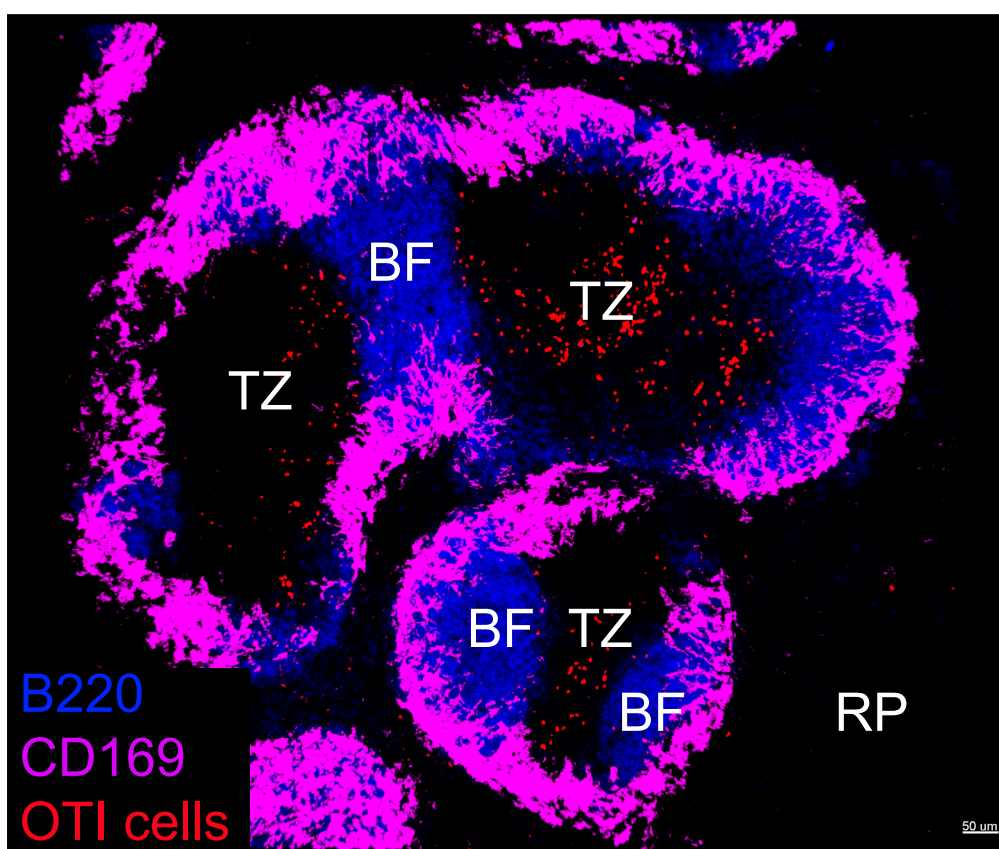
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 484 on mouse lymphocytes: efficacy of inhibitors of protein secretion in protocols for intracellular

485 cytokine staining by flow cytometry. *Journal of Immunology Methods*. **224** (1-2), 69-76 (1999).
486

A



B



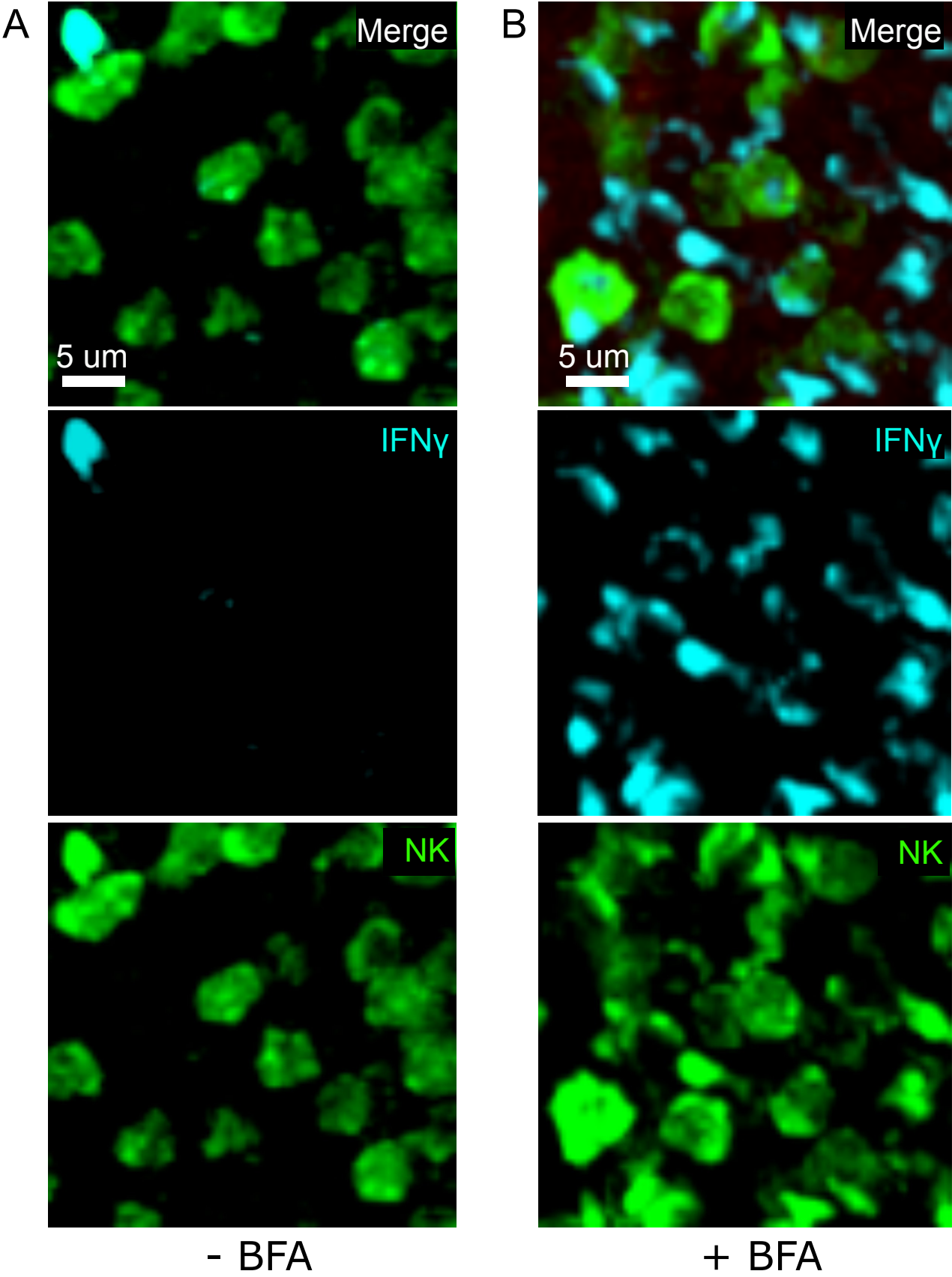
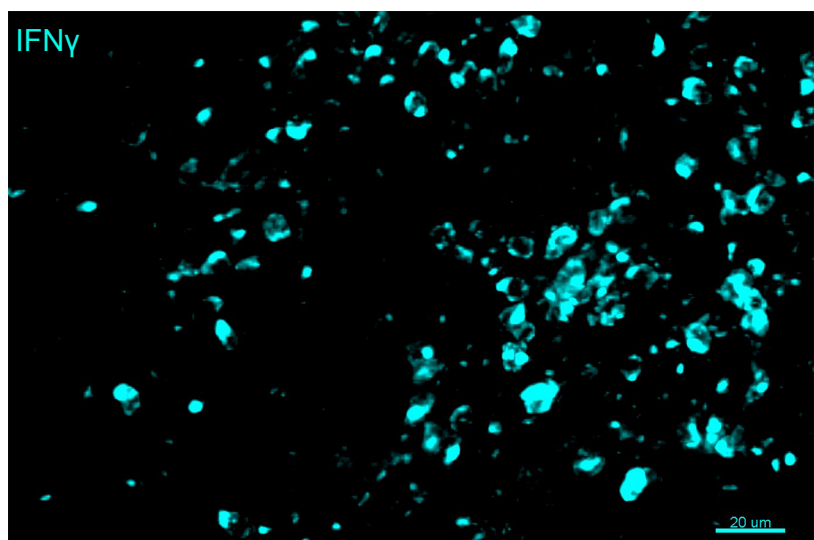
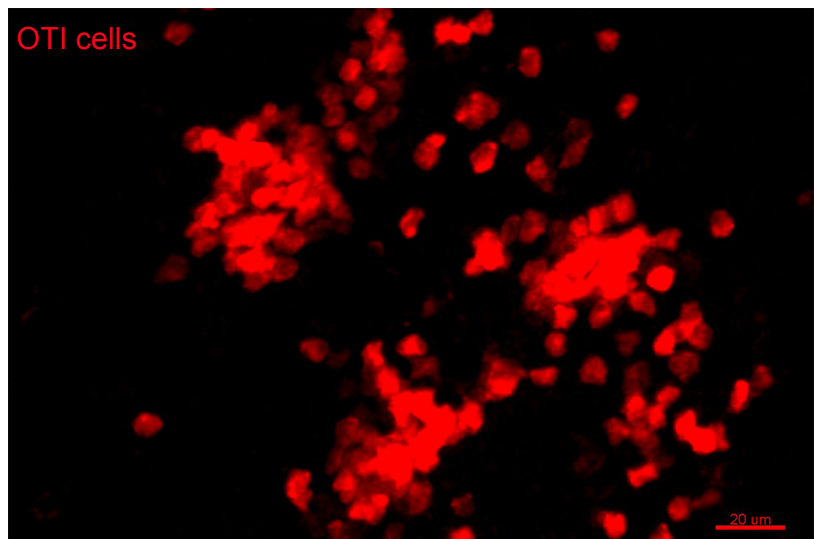
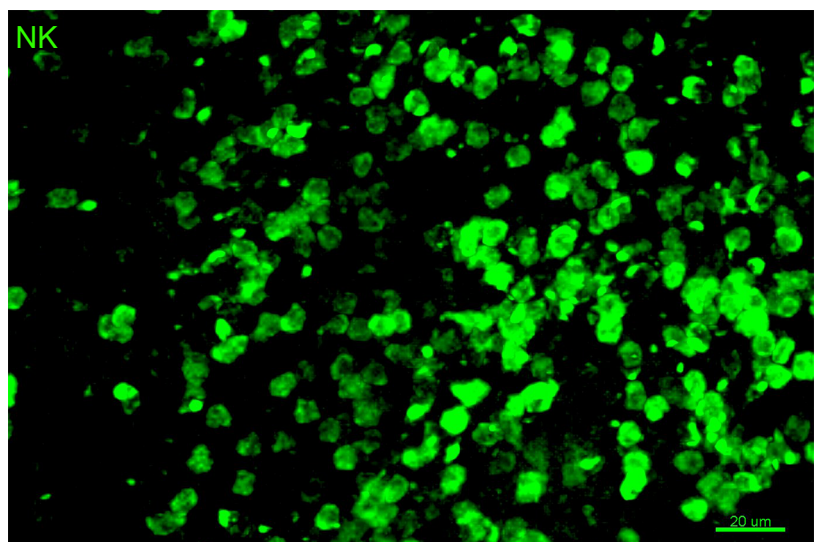
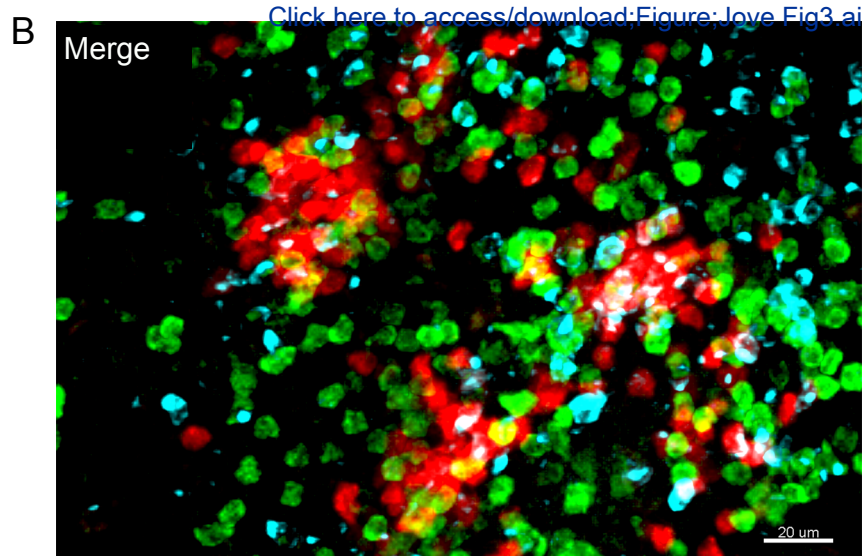
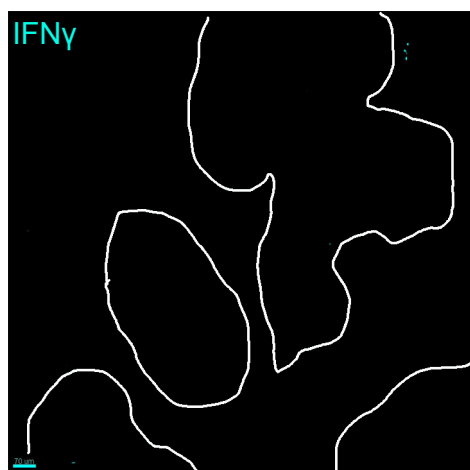
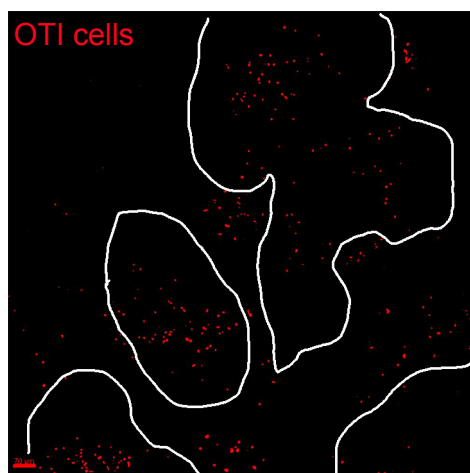
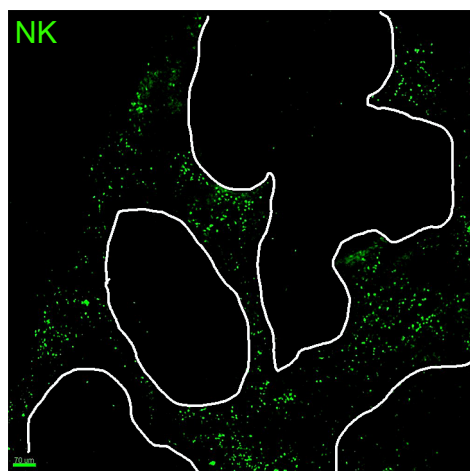
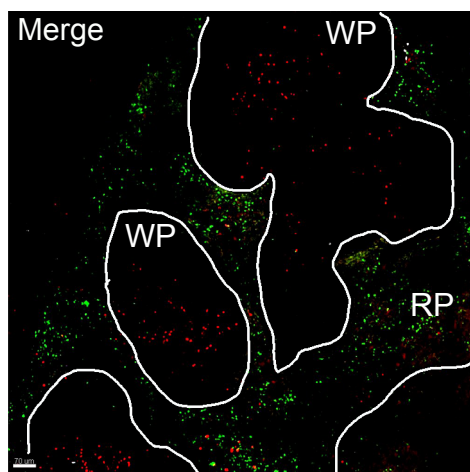
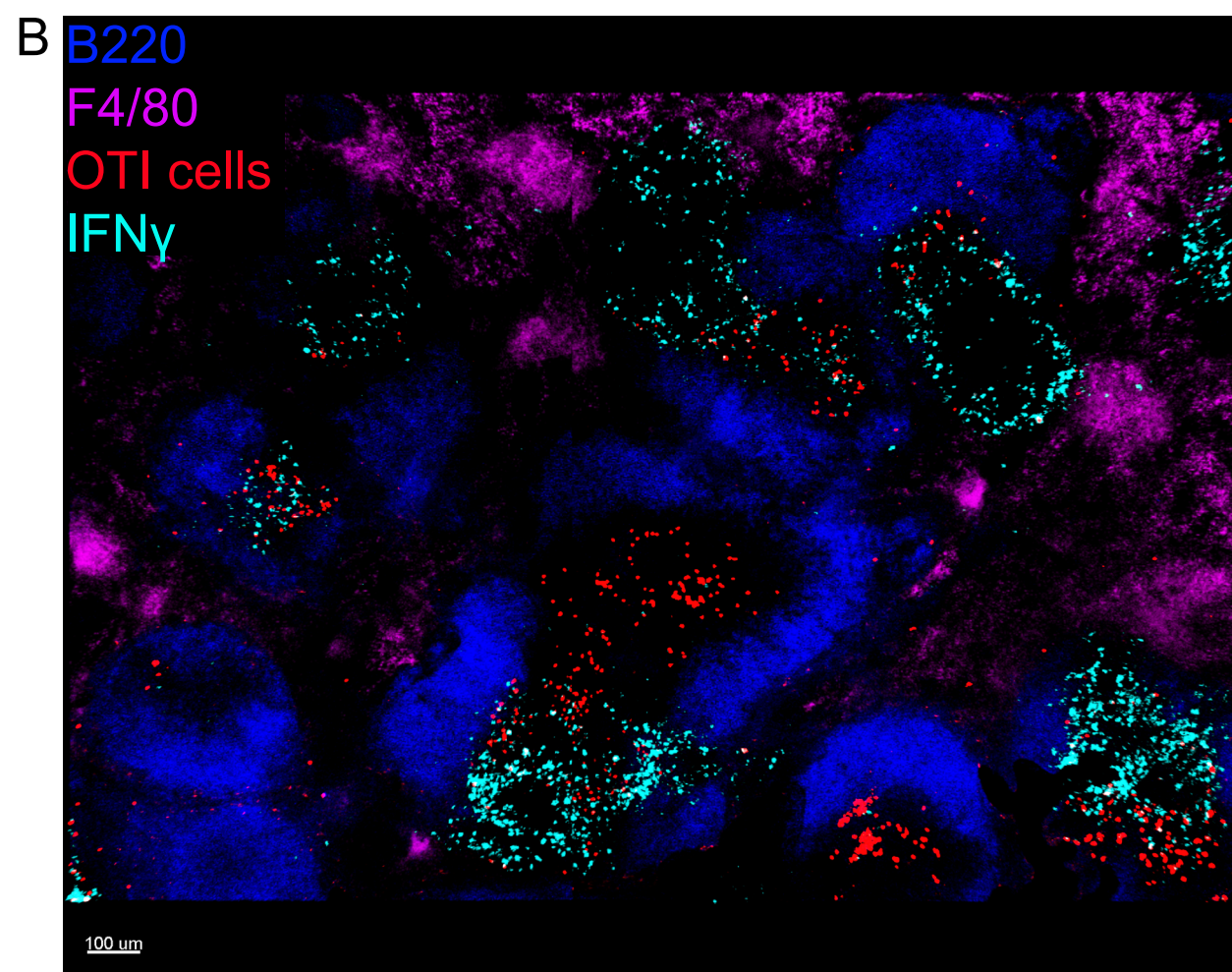
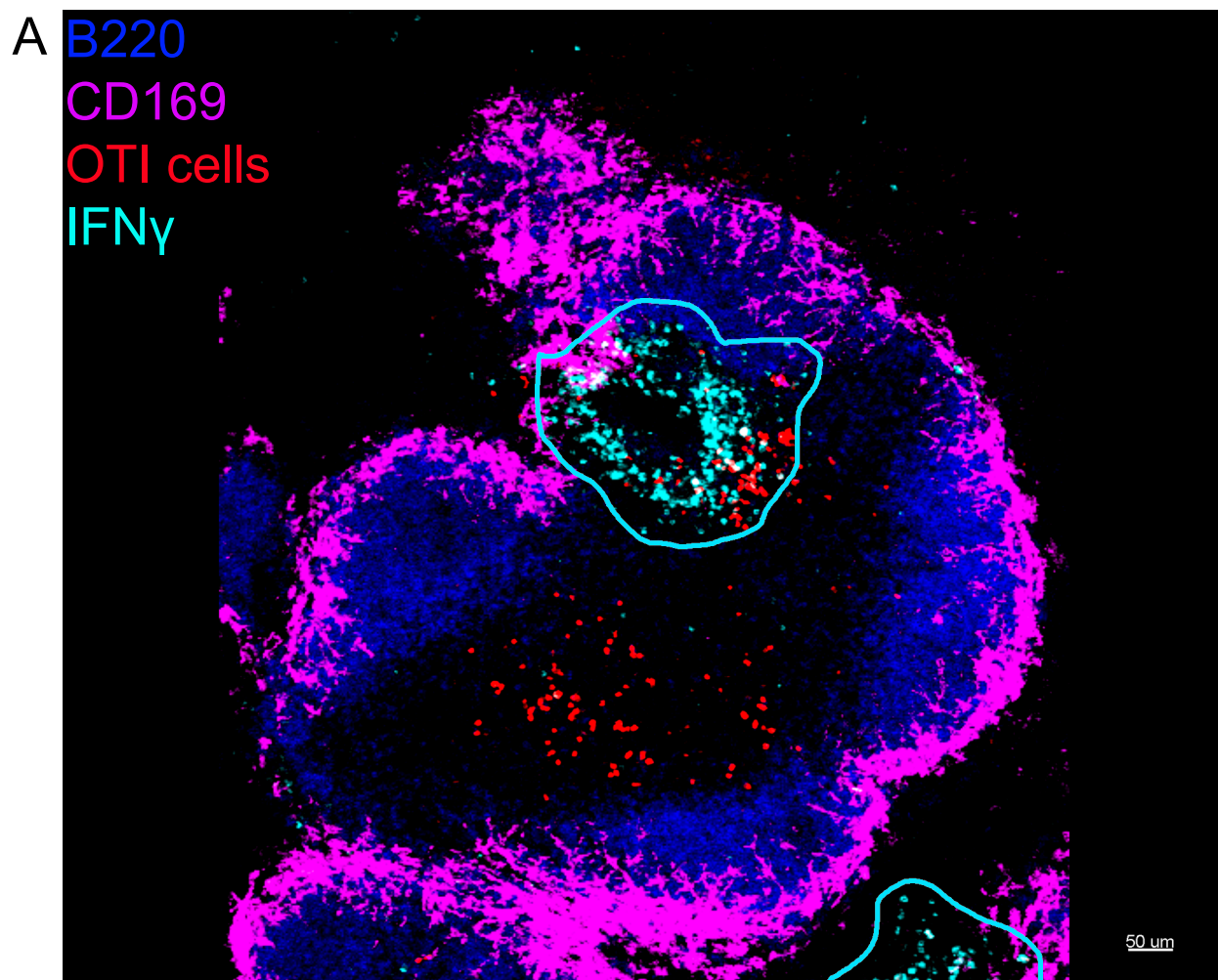


Figure 3

[Click here to access/download:Figure, Iovye Fig3.ai](#)





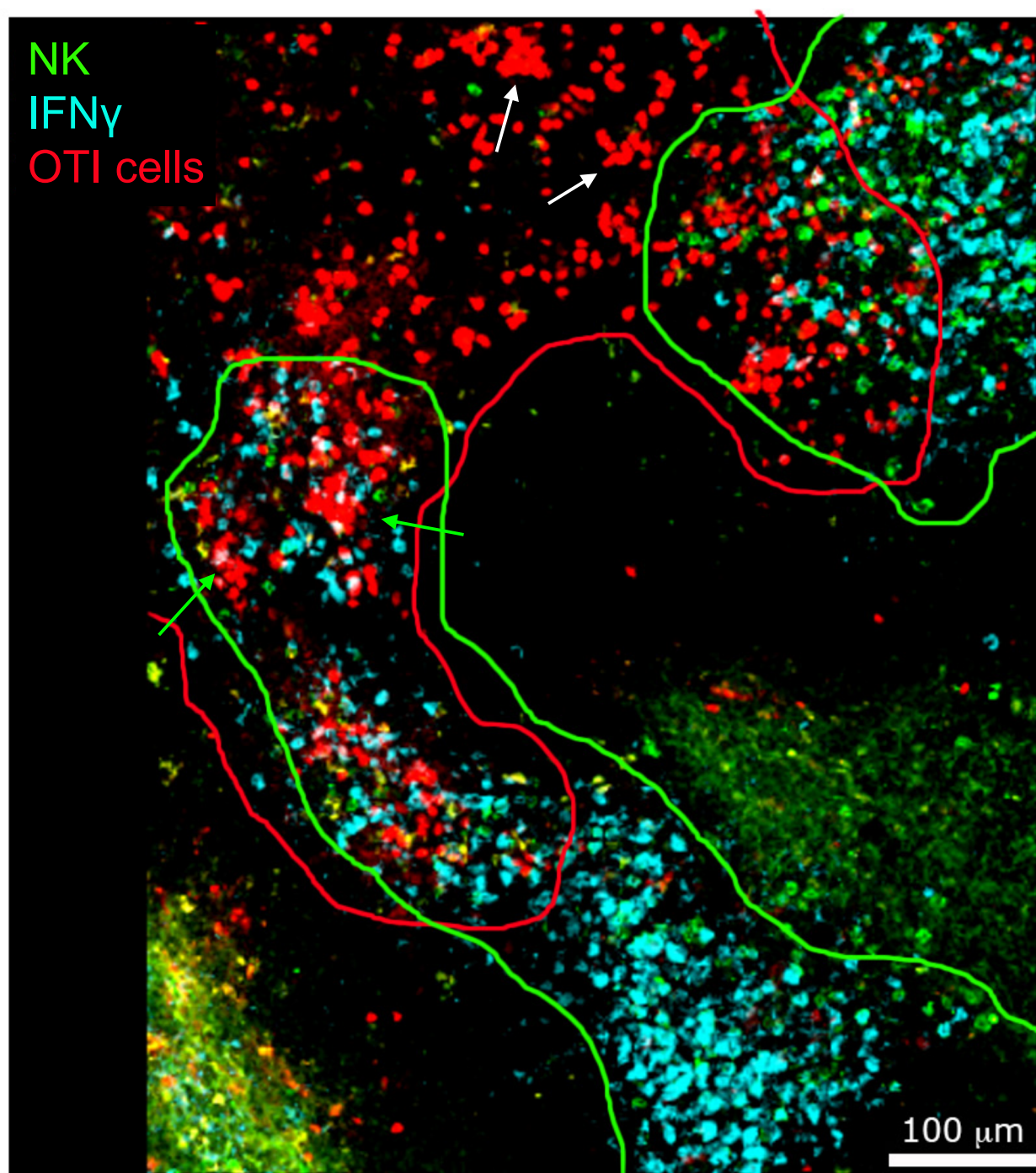
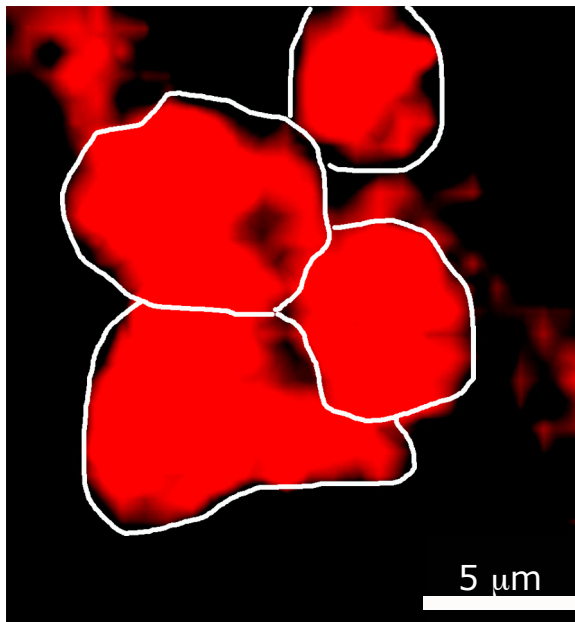
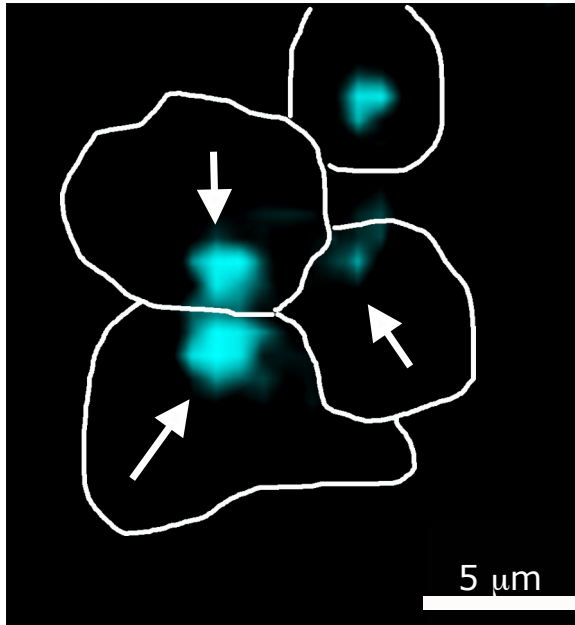
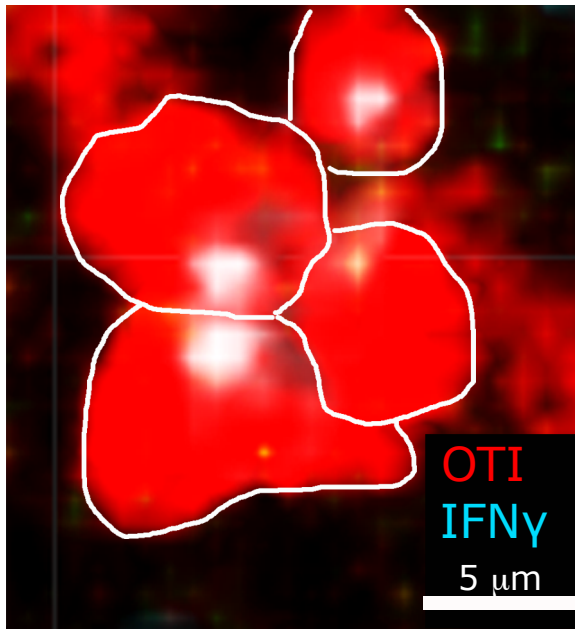
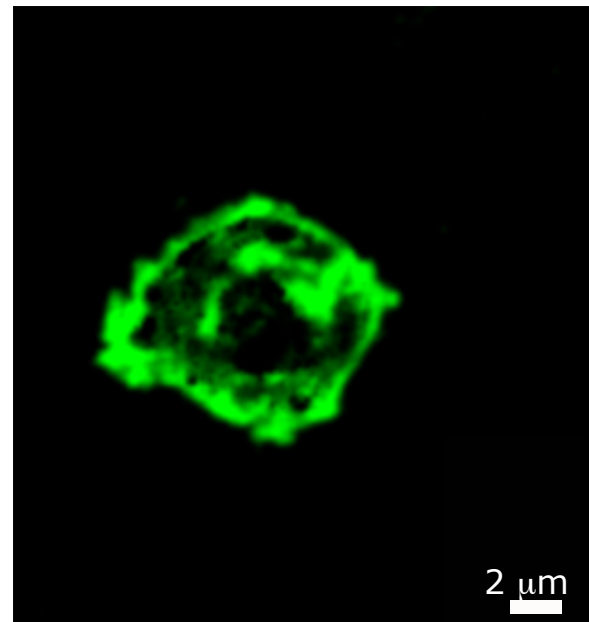
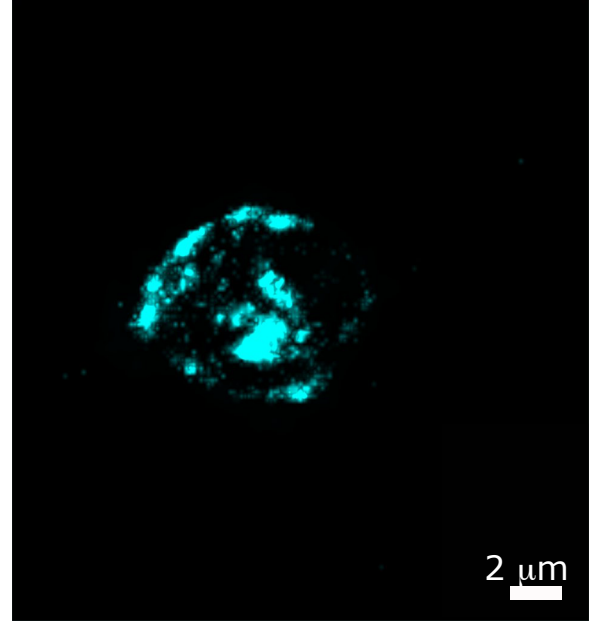
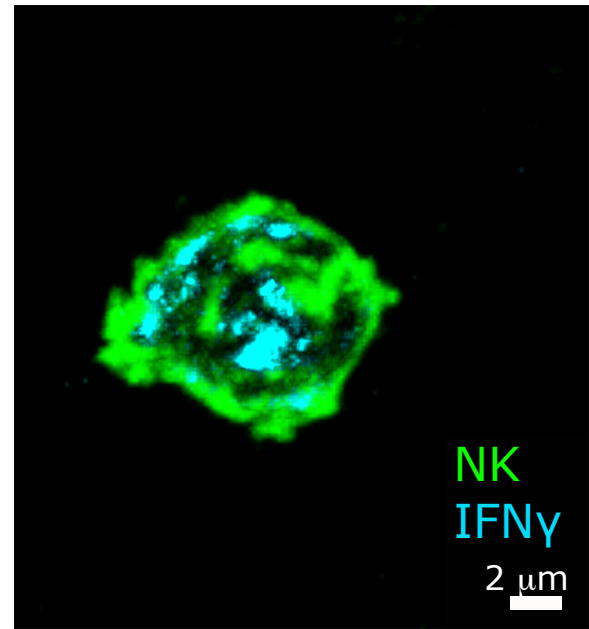


Figure 6

A



B



Name of Material/ Equipment	Company	Catalog Number	Antibody clone and Concentration used		Excitation wavelength	Emission wavelength
Brefeldin A	Cambridge bioscience	CAY11861				
Paraformaldehyde	Agar scientific	R1018				
L-Lysin dihydrochloride	Sigma lifescience	L5751				
Sodium meta-periodate	Thermo Scientific	20504				
D(+)-saccharose	VWR Chemicals	27480.294				
Precision wipes paper Kimtech science	Kimberly-Clark Professional	75512				
O.C.T. compound, mounting medium for cryotomy	VWR Chemicals	361603E				
Fc block, purified anti-mouse CD16/32, clone 93	Biolegend	101302	2.5 µg/ml			
Microscope slides - Superfrost Plus	VWR Chemicals	631-0108				
anti-CD169 - AF647	Biolegend	142407	clone 3D6.112	1.6 µg/ml	650	665
anti-F4/80 - APC	Biolegend	123115	clone BM8	2.5 µg/ml	650	660
anti-B220 - PB	Biolegend	103230	clone RA3-6B2	1.6 µg/ml	410	455
anti-IFNγ - biotin	Biolegend	505804	clone XMG1.2	5 µg/ml		
anti-IFNγ - BV421	Biolegend	505829	clone XMG1.2	5 µg/ml	405	436
anti-Nkp46/NCRI	R&D Systems	AF2225	goat	2.5 µg/ml		
anti-goat IgG-FITC	Novusbio	NPp 1-74814		1 µg/ml	490	525
Streptavidin - PE	Biolegend	405203		2.5 µg/ml	565	578
streptavidin - FITC	Biolegend	405201		2.5 µg/ml	490	525
Fluoromount G	SouthernBiotech	0100-01				
Cover glasses 22x40mm	Menzel-Glazer	12352128				
Liquid blocker super PAP PEN mini	Axxora	CAC-DAI-PAP-S-M				
Imaris - Microscopy Image Analysis Software	Bitplane					
Confocal microscope - Olympus FV1200 Laser scanning microscope	Olympus					

Cryostat - CM 1900 UV	Leica			
Base mould disposable	Fisher Scientific UK Ltd	11670990		
PBS 1X	Life Technologies Ltd	20012068		
BHI Broth	VWR Brand	303415ZA		
GFP			484	507
RFP			558	583
Insulin syringe, with needle, 29G	VWR International	BDAM324824		
C57BL/6 wild type mice	Charles River			



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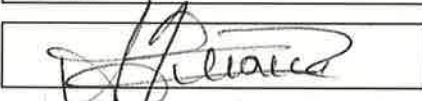
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Response to Editors

Please find below the edits suggested. We tracked all changes in the text, and we edited the figures accordingly.

1- We clarified the method to harvest the spleen as such: "Cleanse the abdomen with 70% ethanol, make an incision with scissors to cut the skin on 1-2cm on the left flank of the mouse, where the spleen is located. Carefully make an incision in the peritoneum to expose the spleen and take it out with tweezers. Harvest the spleen, being careful not to squeeze it with forceps or cut it to avoid disrupting the spleen architecture."

2- We replaced "phosphate buffer" by PBS as suggested.

3- We corrected the few unclear figures legends for more clarity.

4- We edited IFN γ by IFN γ in all figures.