Evaluating the impact of hydroxychloroquine on mouse lymphocyte proliferation and cytokine production in vivo and in vitro

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Protocol
Evaluating the impact of hydroxychloroquine on mouse lymphocyte proliferation and cytokine production in vivo and in vitro

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SUMMARY
Immunomodulatory drugs can alter lymphocyte function. Hydroxychloroquine (HCQ) is prescribed for many autoimmune diseases and is under investigation as an anti-tumor autophagy inhibitor. Here, we describe a protocol to evaluate the influence of HCQ on lymphocyte function by measuring the in vitro and ex vivo proliferation and cytokine production. The protocol can provide insights into potential immunomodulatory effects of HCQ and can be used for assessing other medications' effects on lymphocyte functions. For complete details on the use and execution of this protocol, please refer to Wabitsch et al. (2021).

BEFORE YOU BEGIN
An animal protocol has to be approved according to institutional guidelines. Experience in animal handling, especially the technique of oral gavage in mice for hydroxychloroquine administration and a basic knowledge of flow cytometry is necessary. Additionally, key reagents should be ready before starting the experiment, cell isolation procedure should be performed efficiently to avoid cell death.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Brilliant Violet (BV) 605 rat anti-mouse CD3 (clone 17A2) | BioLegend | 100201 |
| Pacific Blue (PB) rat anti-mouse CD8 (clone 53-6.7) | BioLegend | 100725 |
| Alexa Flour (AF) 700 rat anti-mouse CD4 (clone GK1.5) | BioLegend | 100401 |
| APC rat anti-mouse IFNγ (clone XMG1.2) | BioLegend | 505809 |
| PE rat anti-mouse TNF-α (clone MP6-XT22) | BioLegend | 506305 |
| LIVE/DEAD Near IR Dead Stain Kit | Invitrogen | 2138386 |
| Recombinant Mouse IL-2 | BioLegend | 575404 |
| Bovine Serum Albumin | Sigma | A9647 |
| 2-Mercaptoethanol | Sigma | M3148 |
| Penicillin Streptomycin (P/S) | Gibco | 15140122 |

(Continued on next page)
**Lymphocyte media**

| Reagent                  | Final Concentration | Amount    |
|--------------------------|---------------------|-----------|
| RPMI                     | N/A                 | 500 mL    |
| FCS                      | 10%                 | 50 mL     |
| P/S                      | 1%                  | 5 mL      |
| 2-Mercaptoethanol        | 50 μM               | 2.5 μL    |
| Sodium Pyruvate          | 100 μM              | 500 μL    |

**FACS buffer**

| Reagent                  | Final Concentration | Amount    |
|--------------------------|---------------------|-----------|
| PBS (10x)                | 1x                  | 100 mL    |
| FCS                      | 2%                  | 20 mL     |
| Sodium Azide             | 0.1%                | 10 mL     |
| ddH₂O                    | n/a                 | 870 mL    |
| Total                    | n/a                 | 1000 mL   |

**Extracellular antibody master mix**

| Antibody                  | Dilution Factor |
|---------------------------|-----------------|
| BV 605 anti-mouse CD3     | 1:100           |
| PB anti-mouse CD8         | 1:500           |
| AF700 anti-mouse CD4      | 1:300           |

Note: The addition of P/S is not necessary for the PMA/ionomycin stimulation with a stimulation time of 4hs. However, we did not observe differences in CD4+ and CD8+ T cell activation. The prepared lymphocyte media maybe stored at 4°C for six months.
**Note:** The antibodies for the extracellular staining are diluted in FACS buffer, the antibodies for the intracellular staining were diluted in BD Bioscience Fix/Perm. The FACS buffer maybe stored at 4°C for six months. The antibody master mixes should be prepared and used the day of the experiment.

**STEP-BY-STEP METHOD DETAILS**

**Mouse treatment and single cell suspension of splenocytes**

© Timing: 6 days

For this protocol, we will describe the treatment of mice and splenocyte isolation for further analysis of lymphocyte functions.

1. Treatment of 8 weeks old mice with hydroxychloroquine by daily gavage
   a. Prepare 5 mg/mL HCQ in deionized water and gavage mice with 100 μL daily
   b. On the 6th day, euthanize mice and harvest spleens following institutional animal guidelines. The spleens should be kept in 1× PBS on ice until further steps.
2. Create a single cell suspension of splenocytes by the following steps.
   a. Place the spleen in a 6 well plate with some 1× PBS to keep spleen wet.
   b. Prepare a 50 mL tube with a 5 cm × 5 cm square of 40 μm nylon mesh.
   c. Homogenize the spleen with the end of a plunger from a 5 mL syringe, tamp the spleen against the bottom of the 6 well plate.
   d. Add 10 mL of 1× PBS to the well of the plate in step 2 to resuspend.
   e. With a 10 mL serological pipette, filter the splenocyte solution through the nylon mesh into the 50 mL tube.
   f. Centrifuge at 400 g for 7 min at 4°C.
   g. Aspirate the supernatant.
   h. Resuspend the pellet in 2 mL of ACK lysing buffer for 3 min at 25°C.
   i. Add 10 mL of 5% FCS in 1× PBS to the RBC-lysed splenocyte solution.
   j. Centrifuge at 400 g for 7 min at 4°C.
   k. Aspirate the supernatant and resuspend the splenocytes in 5 mL of 1× PBS.
   l. Gather a new 15 mL tube and a 5 cm × 5 cm square of 40 μm nylon mesh.
   m. Filter the splenocyte solution through the nylon mesh into the 15 mL tube.

**Optional:** To distinguish the effects of treatment on lymphocyte function in different organs you can also harvest the liver, lymph nodes or blood from the mice. To create a single cell suspension of hepatic lymphocytes please refer to (Yoneyama et al., 1998), for lymph nodes lymphocytes to (Lim et al., 2016) and for blood lymphocyte separation to (Spranger et al., 2010).

**Optional:** For additional investigation of cell exhaustion upon HCQ or other treatments you can add anti-PD-1, Tim3 and CD69 to the extracellular master mix and stain unstimulated lymphocytes.

### Extracellular antibody master mix

| Diluent          | Volume per sample |
|------------------|-------------------|
| FACS Buffer      | 150 μL            |

### Intracellular antibody master mix

| Diluent          | Volume per sample |
|------------------|-------------------|
| BD Bioscience Fix/Wash buffer | 150 μL            |
Treatment of stimulated lymphocytes

**Timing:** 8 h

Here we describe the PMA/ionomycin stimulation experiment. For this experiment use splenocytes previously isolated from either HCQ treated or untreated mice.

3. Key step: count splenocytes to ensure the final number of cells per well.
4. Take desired cell number and spin down at 400 g, 4°C, for 7 min and discard the supernatant
5. Dilute splenocyte at a final concentration of $2 \times 10^6$ splenocytes/mL in lymphocyte media
6. Add 100 UI/mL (final concentration) of IL-2 to lymphocyte media to make completed lymphocyte media
7. Seed 100 µL of cell suspension in 96 V-bottom plate

**Pause point:** Use splenocytes from untreated mice to test the effects of in vitro HCQ treatment on splenocytes.

8. Prepare the treatment media by adding HCQ (molecular mass 319 g/mol) to completed lymphocyte media as shown in Table 1.

9. Add 50 µL of the HCQ treatment media to each well of the splenocytes of untreated mice and use technical triplicates for every HCQ concentration.
10. Prepare the stimulation media by adding 8 µL/mL stimulation mix (PMA/ionomycin)
11. Add 50 µL of stimulation media to in vivo HCQ treated splenocytes or in vitro HCQ treated splenocytes as well as controls (the final concentration of stimulation mix is 2 µL/mL). Use one sample as unstimulated control.

**Pause point:** Now you have in vitro stimulated splenocytes in 200 µL, splenocytes from in vivo treated mice in 150 µL and unstimulated controls in 100 µL (Figure 1).

12. Add 50 µL completed lymphocyte media to splenocytes of treated mice and 100 µL to the unstimulated controls
13. Incubate splenocytes in 37°C for 4 h
14. Spin down plate at 400 g, 4°C, for 7 min and discard the supernatant
15. Wash once with FACS buffer and discard the supernatant

**Critical:** If you want to adapt this protocol, avoid centrifuge steps and other manipulations before the incubation of splenocytes to minimize the risk of cell death and improve cytokine signaling.

**Optional:** Additional marker for cell exhaustion such as anti-PD-1, Tim3 and CD69 could be added to this panel to further investigate the influence of medications on lymphocytes.

16. Stain for flow cytometry analysis

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**Table 1. Calculation of HCQ concentrations**

| HCQ µg/mL | Final Concentration |
|-----------|---------------------|
| 6.38      | 20 µM               |
| 12.76     | 40 µM               |
| 25.52     | 80 µM               |
| 51.01     | 160 µM              |

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a. Prepare Live/Dead (L/D) stain by adding 1 μL/mL L/D stain to PBS
b. Resuspend cells in 50 μL L/D stain and incubate for 20 min in 4°C
c. Add 150 μl FACS buffer to each well and spin down plate at 400 g, 4°C, for 7 min and discard the supernatant
d. Resuspend cells in 50 μL extracellular antibody master mix and incubate for 15 min in the dark at RT.
e. Add 150 μl FACS buffer to each well and spin down plate at 400 g, 4°C, for 7 min and discard the supernatant
f. Resuspend cell in 100 μL of BD Bioscience Fix/Perm and incubate at 4°C in the dark for 30 min
g. Spin down plate at 400 g, 4°C, for 7 min and discard the supernatant
h. Resuspend cells in 50 μL intracellular antibody master mix and incubate for 15 min in the dark in RT
i. Add 150 μl FACS buffer to each well and spin down plate at 400 g, 4°C, for 7 min and discard the supernatant
j. Dilute cells in 150 μl FACS Buffer for analysis for flow cytometry analysis

17. Analyze data using FlowJo™ software

Optional: To get a better insight of the lymphocyte cytokine production profile, different concentrations of the stimulation mix can be applied. The recommended final concentration is 2 μL/mL but we observed positive TNFα and IFNγ signaling starting from a concentration of 0.5 μL/mL.

Proliferation and cytokine production of treated lymphocytes after antigen-specific activation

@ Timing: 2–3 days

For this protocol we describe how to evaluate antigen-specific cytokine production as well as lymphocyte proliferation using CFSE stained OT-1 lymphocytes.
18. Sacrifice one 8 weeks old OT-1 mouse following institutional animal guidelines and harvest the spleen.
19. Create a single cell suspension as described in step 2.
20. Resuspend 5 x 10^7 splenocyte cells in 10 mL prewarmed 0.1% BSA/PBS in 50 mL tube.
21. Add 5 μL of a 5 mM stock solution CFSE on the wall of the 50 mL tube and vortex.
22. Put the 50 mL tube in a water bath 37°C for 6 min.
23. Stop CFSE staining of cells by adding 25 mL FSC.
24. Centrifuge the tube at 400 g, 4°C, for 7 min and discard the supernatant.
25. Wash cells once with completed media.
26. Resuspend cells in completed lymphocyte media (see step 6) and resuspend cells with a final concentration of 2 x 10^6 splenocytes/mL.
27. Seed 100 μL per well of the cell suspension in a 96 well flat bottom plate.
28. Prepare treatment media as described in step 7.
29. Prepare stimulation media by adding 4 μg/mL OVA-I peptide to completed lymphocyte media (final concentration 1 μg/mL).
30. Add 50 μL of stimulation media to splenocytes. Use one sample as unstimulated control.
31. Add 50 μL of treatment media to splenocytes.

Note: In this experiment, it is crucial to add P/S to the media as the incubation time is longer and the risk for infection higher. Again, avoiding centrifuge steps until the incubation improves viability of the cells and consistency of the results.

32. Incubate splenocytes in 37°C for 2 days.
33. Spin down plate at 400 g, 4°C, for 7 min and discard the supernatant.
34. Save the supernatant to perform ELISA analysis following vendor’s instructions.
35. Wash pelleted by adding 5 mL PBS, centrifuge at 400 g, 4°C, for 7 min and discard the supernatant.
36. Perform an extracellular staining of cells for flow cytometry analysis as described in Step 15. Dilution of CFSE can be detected with the FITC channel.

EXPECTED OUTCOMES

There is very low TNFα and IFNγ signal in the unstimulated samples (gating strategy for CD8+ T cells is shown in Figure 2A); the negative samples are needed to set the negative gate. There should be a robust signal of TNFα and IFNγ in the stimulated control samples (Figure 2B). The signal depends on cell viability which should be over 85% as well as the concentration of the stimulation cocktail (Figure 2B). Typical results of IFNγ and TNFα production of untreated (control) CD8+ T cells stimulated with 1 and 2 μL/mL stimulation cocktail are shown in Figures 2C and 2D. The shapes of between lymphocytes from 4h PMA/ionomycin stimulation and 48h OT-1/OVA-I peptide stimulation are different. Lymphocytes gain size after 48h of OT-1/OVA-I stimulation and the gating must be set correctly (Figure 2F). For CFSE dilution, unstimulated lymphocytes serve to set the negative gate (Figure 2G). For further information of the results of HCQ treatment please refer to (Wabitsch et al., 2021).

LIMITATIONS

First, this protocol does not measure the serum concentration of HCQ in mice, also the lymphocyte functional study relies on in vitro or ex vivo assays, therefore the effective serum HCQ concentration on lymphocyte functional regulation cannot be determined. Further pharmacokinetic studies of HCQ in mice, with the corroboration of in vivo lymphocyte functional assays, will help gain a in depth understanding of how HCQ influences lymphocyte functions.

Second, the protocol was designed for CD8+ and CD4+ T cells and conditions may not be optimal for other lymphocytes. For example, NKT cells quickly internalize their T cell receptor within hours upon in vitro stimulation, which makes them undetectable by tetramer staining. Therefore, this protocol...
should be optimized to cell types wherein tetramer staining is needed for cell definition in flow cytometry.

**TROUBLESHOOTING**

**Problem 1 (step 17)**

No cytokine signal in flow cytometry or ELISA analyses

**Potential solution**

Cell death is the most probable reason for the lack of cytokine signal. Avoiding centrifuge steps before incubation and cell culture contamination is crucial. Additionally, this protocol uses $1 \times 10^5$ cells per
well. A cell number per well of more than $1 \times 10^6$ impacts the cytokine production. If high cell number is needed, a 24 well plate and dilution in a higher volume improves the outcome. Increasing stimulation cocktail concentration may be necessary if cellular death is excluded; but should PMA/ionomycin stimulation mix concentration not exceed 6 μL/mL.

**Problem 2 (step 17)**
No difference in lymphocyte function is apparent between treatment and non-treatment groups

**Potential solution**
The impact of many immunomodulatory drugs on lymphocyte functions can be evaluated using this protocol. Yet because of the in vitro and ex vivo setting of these experiments, these assays may not detect all in vivo changes in lymphocyte function. If a treatment difference on lymphocyte function is expected, you should ensure adequate serum concentration and evaluate the treatments pharmacokinetics and pharmacodynamics. A reason to further investigate these potential challenges is an obvious difference between in vivo and in vitro treatment conditions.

**Problem 3 (step 26)**
Cell death during 48 h antigen-specific stimulation

**Potential solution**
Decreasing the number of centrifuge steps and other manipulations and adding P/S to the media are important to avoid cell culture infection. If necessary, the treatment media may be changed once. Additionally, cell death especially occurs in the unstimulated CD8- T cell fraction which is not stimulated by the OVA-I peptide. This can be evaluated by L/D staining of CD8+ and CD8- T cells.

**Problem 4 (step 34)**
Sample well cytokine signal is not within the standard curve range of ELISA

**Potential solution**
We recommend using a 1:10 dilution of sample supernatant for TNFα and IFNγ ELISAs. However, this dilution may not be inadequate to properly interpolate the standard curve of respective ELISA. Sample dilutions should be adjusted and optimized accordingly.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Tim Greten, tim.greten@nih.gov

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
The data that supports the findings of this study are available upon reasonable request.

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AUTHOR CONTRIBUTIONS
Project design: S.W., C.M., and T.G.; completion of experiments: S.W. and J.D.M.; data analysis and interpretation: S.W., J.D.M., C.M., and T.F.G.; writing original draft: S.W., J.D.M., and T.F.G.; and review and editing: all authors.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
Lim, J.F., Berger, H., and Su, I.H. (2016). Isolation and activation of murine lymphocytes. J. Vis. Exp. 116. https://doi.org/10.3791/54596.

Spranger, S., Javorovic, M., Burdek, M., Wilde, S., Mosetter, B., Tippmer, S., Bigalke, I., Geiger, C., Schendel, D.J., and Frankenberger, B. (2010). Generation of Th1-polarizing dendritic cells using the TLR7/8 agonist CL075. J. Immunol. 185, 738–747.

Wabitsch, S., Movey, J.C., Ma, C., Ruf, B., Kamenyeva, O., Mccallen, J.D., Diggs, L.P., Heinrich, B., and Greten, T.F. (2021). Hydroxychloroquine can impair tumor response to anti-PD1 in subcutaneous mouse models. iScience 24, 101990.

Yoneyama, H., Harada, A., Imai, T., Baba, M., Yoshe, O., Zhang, Y., Higashi, H., Murai, M., Asakura, H., and Matsushima, K. (1998). Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. J. Clin. Invest. 102, 1933–1941.