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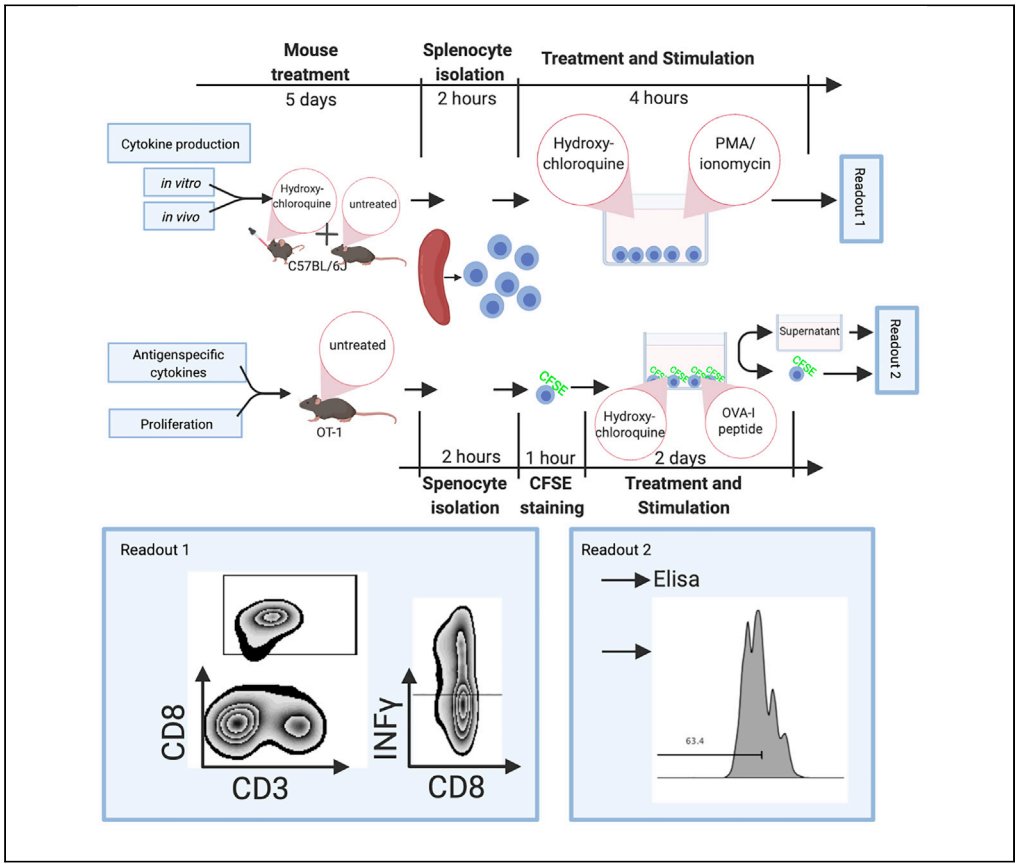
Evaluating the impact of hydroxychloroquine on mouse lymphocyte proliferation and cytokine production *in vivo* and *in vitro*



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.

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Highlights

Evaluate the in vitro and ex vivo effects of HCQ on mouse lymphocyte function

Assess the immunomodulatory features of hydroxychloroquine

Experimental framework to assess the immunomodulatory features of other reagents

Studies of lymphocyte function can be completed within 10 days

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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
Chemicals, peptides, and recombinant proteins		
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

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sodium Pyruvate	Gibco	11360070
ACK Lysing Buffer	Quality Biological	118156101
OVA Peptide I	Invivogen	257264
Hydroxychloroquine (HCQ)	NIH Clinical Pharmacy	6722121
Critical commercial assays		
Mouse TNFa ELISA Kit	Abcam	ab100747
Mouse IFNγ ELISA Kit	Abcam	ab100689
Experimental models: organisms/strains		
Mouse: wild type (WT): C57BL/6J	Charles River	556
Mouse: OT 1	Charles River	C57BL/6-Tg(TcraTcrb)1100Mjb/Crl
Software and algorithms		
FlowJo v10.6	FlowJo	N/A
Other		
Fixation and Permeabilization Buffer	BD	554722
PMA Ionomycin with Golgi Block	BD	550583
CellTrace CFSE Cell Proliferation Kit	Thermo Fisher	C34554
Fetal Calf Serum (FCS)	Sigma	12306C
Sodium Azide	Sigma	S2002
RPMI 1640 Medium, GlutaMAX	Gibco	61870036

MATERIALS AND EQUIPMENT

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

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.

FACS buffer		
Reagent	Final Concentration	Amount
PBS (10×)	1×	100 mL
FCS	2%	20 mL
Sodium Azide	0.1%	10 mL
ddH_2O	n/a	870 mL
Total	**n/a**	**1000 mL**

Extracellular antibody master mix	Dilution Factor
BV 605 anti-mouse CD3	1:100
PB anti-mouse CD8	1:500
AF700 anti-mouse CD4	1:300

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Extracellular antibody master mix	Dilution Factor
Diluent	Volume per sample
FACS Buffer	150 μL
Intracellular antibody master mix	Dilution Factor
APC anti-mouse IFNγ	1:200
PE anti-mouse TNF- α	1:200
Diluent	Volume per sample
BS Bioscience Perm/Wash buffer	150 μL

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.

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

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 2×10^6 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

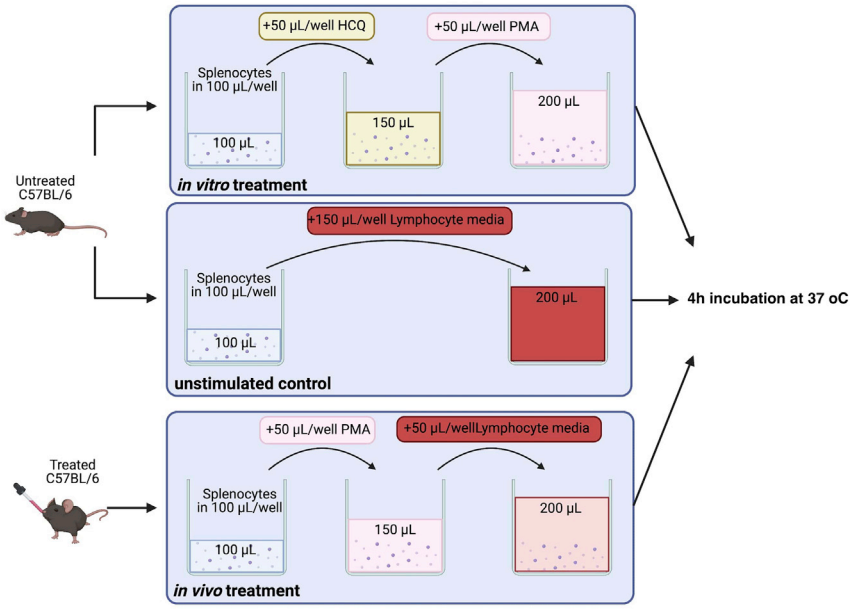

Figure 1. Illustration of treatment of splenocytes from treated and untreated mice
L/D, Live/Dead

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 100µL 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 5×10^7 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 2×10^6 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 pellet once 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

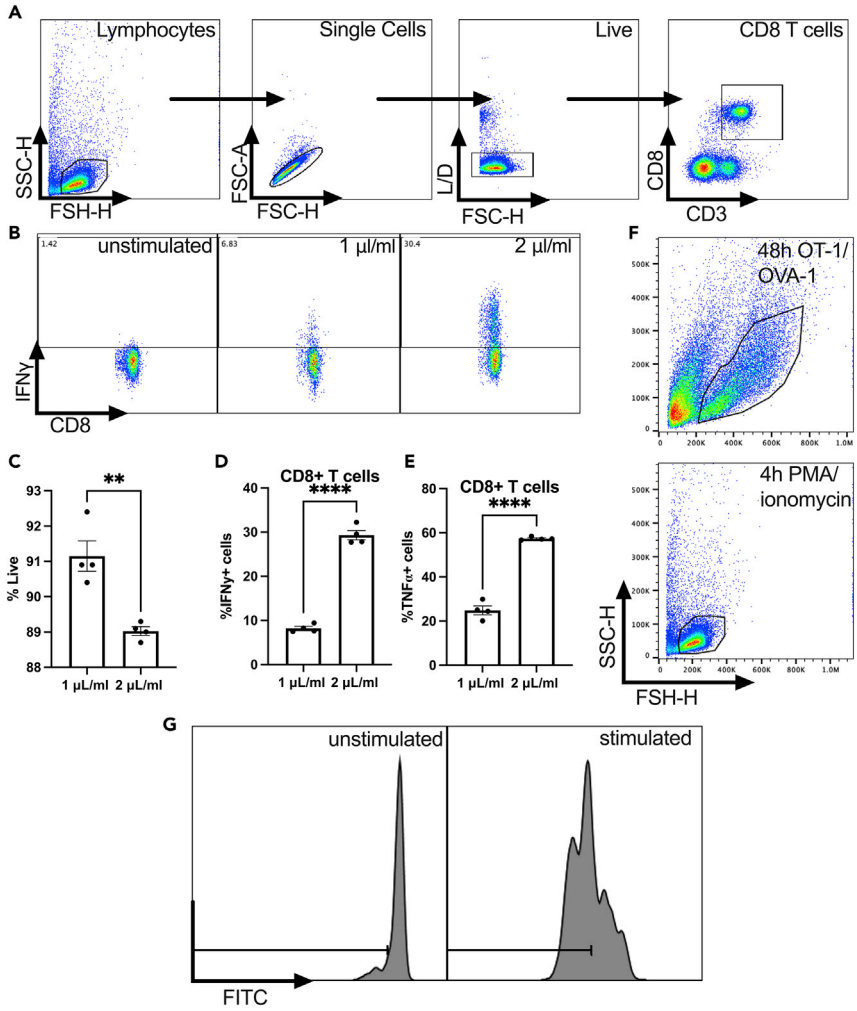

Figure 2. Analysis of flow cytometry data and IFNγ signal after stimulation

(A) Representative contour plot of the gating strategy for $CD8^+$ T cells (Lymphocytes/singlets/Live cells) and gating for IFNγ+ of unstimulated and stimulated splenocytes with 1 and 2 µL/mL of stimulation cocktail.

(B) Percentage of live splenocytes after in vitro stimulation for 4 h with 1 (n=4) and 2 (n=4) µL/mL of stimulation cocktail. **p<0.01; student's t-test.

(C and D) Percentage of TNFα (C) and IFNγ (D) $CD8^+$ T cells after in vitro stimulation for 4 h with 1 (n=4) and 2 (n=4) µL/mL of stimulation cocktail. ****p<0.0001; student's t-test.

(E) Representative contour plot of the gating strategy for lymphocytes after 2 µL/mL of PMA/ionomcin stimulation for 4 h (left) and OVA-1 peptide (1 µg/mL) stimulation of OT-1 splenocytes for 48 h (right).

(F) Representative contour plot of the gating strategy for CFSE negative OT-1 splenocytes after stimulation with OVA-1 peptide (1 µg/mL) for 48 h (right).

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 1×10^5 cells per

well. A cell number per well of more than 1×10^6 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.

ACKNOWLEDGMENTS

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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.

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