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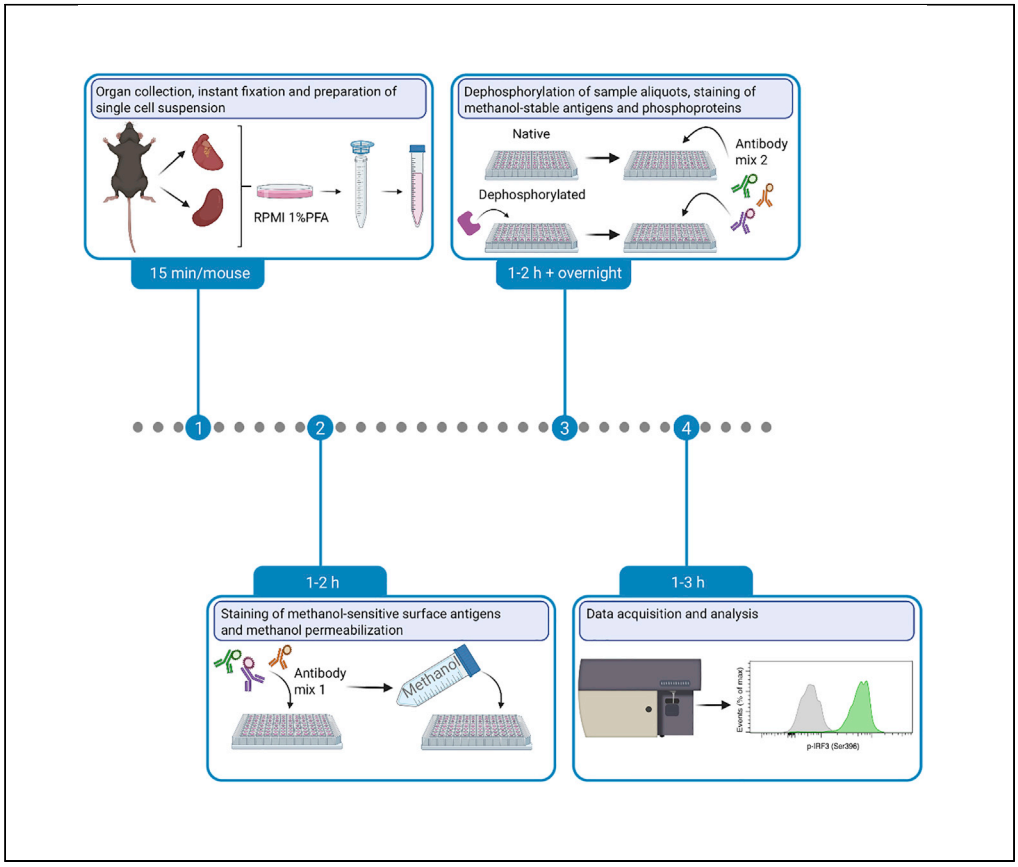
Quantification of unperturbed phosphoprotein levels in immune cell subsets with phosphoflow to assess immune signaling in autoimmune disease



Activation of innate immune sensors by endogenous DNA and RNA can lead to autoimmune and autoinflammatory diseases. Quantification of the unperturbed phosphoprotein content in immune cells provides insight into the spontaneous activity of immune signaling pathways triggered by nucleic acid recognition. Here, we present a phosphoflow protocol for measuring phosphoproteins in mouse models of autoimmunity that incorporates strategies to preserve native phosphoprotein levels during sample collection and to reliably detect low signaling activity common in chronic disease states.

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Highlights

Phosphoflow protocol to quantify basal immune signaling activity

Strategies to preserve native phosphorylation levels and account for background signal

Reliable detection of small changes in phosphoprotein content in chronic diseases

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Quantification of unperturbed phosphoprotein levels in immune cell subsets with phosphoflow to assess immune signaling in autoimmune disease

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SUMMARY

Activation of innate immune sensors by endogenous DNA and RNA can lead to autoimmune and autoinflammatory diseases. Quantification of the unperturbed phosphoprotein content in immune cells provides insight into the spontaneous activity of immune signaling pathways triggered by nucleic acid recognition. Here, we present a phosphoflow protocol for measuring phosphoproteins in mouse models of autoimmunity that incorporates strategies to preserve native phosphoprotein levels during sample collection and to reliably detect low signaling activity common in chronic disease states.

For complete details on the use and execution of this protocol, please refer to Jütte et al. (2021).

BEFORE YOU BEGIN

Design antibody staining panel

When developing a staining panel for phosphoflow, two important aspects have to be considered. First, many fluorophores (e.g., PE, APC) are damaged by methanol permeabilization. Antibodies conjugated to these fluorophores can only be used for staining after methanol treatment. Second, epitopes of certain antigens (e.g., CD19, Ly6C) are destroyed by methanol permeabilization. Staining of these antigens must be performed in a staining step before methanol treatment with antibodies conjugated to methanol-stable fluorophores.

Fluorophores tested for methanol-stability in this protocol

Fluorophore	Effect of methanol on fluorescence intensity
PE	–
FITC	+
Alexa Fluor 488	+
PerCP/Cy5.5	–
PE/Cyanine 7	–
Alexa Fluor 647	+
APC	+/–
APC/Cyanine 7	+/–
Pacific Blue	+

(*Continued on next page*)

Continued	
Fluorophore	Effect of methanol on fluorescence intensity
Brilliant Violet 421	+
Brilliant Violet 510	+/–

+ signal unchanged, +/– signal reduced, – signal lost.

To find out whether an epitope is methanol-sensitive, you can either refer to manufacturer lists or perform pilot experiments.

The protocol below describes how to measure phospho-IRF-3 and phospho-Stat1 but can easily be adopted for other phosphoproteins. IRF-3 is activated by nucleic acid sensors such as cGAS, RIG-I, MDA5 and TLR3. Stat1 signaling is induced by type-I, type-II and type-III interferons.

Note: If the staining panel requires antibodies other than those used in this protocol, appropriate working concentrations must be determined in preliminary tests.

Note: In this experimental setting it is not possible to use viability dyes since cells are immediately fixed after organ harvesting.

Institutional permissions

Experimental procedures were performed in accordance with the German Animal Welfare Act and approved by the State Agency for Nature, Environment and Consumer Protection, NRW.

Before you start using the methods described in this protocol, it is essential that you obtain the necessary permissions from your local regulatory agency.

Prepare antibody mixtures

The antibody mixtures are prepared in staining buffer on the day of the experiment. Store at 4°C protected from light.

1. Prepare the antibody mixture for those surface antigens that need to be stained before methanol permeabilization.

Antibody mix 1				
Antigen	Fluorophore	Clone	Dilution	Company
CD19	Alexa Fluor 647	6D5	1:500	Biolegend
TruStain FcX™ (anti-mouse CD16/32)	N/A	93	1:200	BioLegend

2. Prepare the antibody mixture for antigens that will be stained after methanol permeabilization, including phosphoproteins.

Antibody mix 2				
Antigen	Fluorophore	Clone	Dilution	Company
I-A/I-E	APC/Cyanine 7	M5/114.15.2	1:200	BioLegend
CD8a	Brilliant Violet 421	53-6.7	1:200	BioLegend
CD11b	Brilliant Violet 510	M1/70	1:100	BioLegend
CD11c	PE/Cyanine 7	N418	1:250	BioLegend
TCRß chain	PerCP/Cy5.5	H57-597	1:125	BioLegend
P-IRF-3 (Ser396)	PE	D6O1M	1:25	Cell Signaling Technology
P-Stat1 (Y701)	Alexa Fluor 488	58D6	1:25	Cell Signaling Technology
TruStain FcX™ (anti-mouse CD16/32)	N/A	93	1:200	BioLegend

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor® 647 anti-mouse CD19 clone: 6D5 (1:500)	BioLegend	Cat#115522 RRID: AB_389329
APC/Cyanine 7 anti-mouse I-A/I-E clone: M5/114.15.2 (1:200)	BioLegend	Cat# 107627 RRID: AB_1659252
Brilliant Violet 421™ anti-mouse CD8α clone: 53-6.7 (1:200)	BioLegend	Cat#100753 RRID: AB_2562558
Brilliant Violet 510™ anti-mouse/ human CD11b clone: M1/70 (1:100)	BioLegend	Cat#101263 RRID: AB_2629529
PE/Cyanine 7 anti-mouse CD11c clone: N418 (1:250)	BioLegend	Cat#117318 RRID: AB_493568
PerCP/Cy5.5 anti-mouse TCRß chain clone: H57-597 (1:125)	BioLegend	Cat#109228 RRID: AB_1575173
P-IRF-3 (Ser396) (D6O1M) rabbit mAB (PE Conjugate) (1:25)	Cell Signaling Technology	Cat# 83611S RRID: AB_2800022
P-Stat1 (Y701) (58D6) Rabbit mAB Alexa (R) 488 (1:25)	Cell Signaling Technology	Cat#9174S RRID: AB_2198287
TruStain FcX™ (anti-mouse CD16/32) clone: 93 (1:200)	BioLegend	Cat# 101320 RRID: AB_1574975
Chemicals, peptides, and recombinant proteins		
λ Protein Phosphatase	New England Biolabs	Cat# P0753
Dulbeccoś Phosphate-buffered Saline (PBS)	Gibco	Cat# 14190-094
FBS Superior standardized	Merck	Cat# S0615
Albumin Fraction V	Carl Roth	Cat# 8076.2
Normal rabbit serum control	Invitrogen	Cat# 10510
Paraformaldehyde, 16% w/v aq. soln., methanol free	Thermo Fisher Scientific	Cat# 043368.9
RPMI Medium 1640 (1×) + GlutaMax™-I	Gibco	Cat# 72400-021
Sodium azide pure	AppliChem	Cat# A1430,0100
Experimental models: Organisms/strains		
Mouse: C57Bl6/J, female and male, 8 weeks of age	The Jackson Laboratory	Stock No. 000664
Mouse: B6;129P2-*Trex1tm1Tld (Trex1*$^{-/-}$), female and male, 8 weeks of age	Tomas Lindahl (Cancer Research UK, London)	N/A
Software and algorithms		
FlowJo V10	BD Biosciences	https://www.flowjo.com
GraphPad Prism 8	GraphPad Software	https://www.graphpad.com
BioRender	BioRender	https://biorender.com
Other		
BD FACSCanto II	BD Biosciences	https://www.bdbiosciences.com/
Nitex membrane 70 µM mesh	Klein & Wieler oHG	N/A
96-Well micro test plates, U-bottom, PP	Ratiolab	Cat# 6018121

MATERIALS AND EQUIPMENT

Staining buffer		
Reagent	Final concentration	Volume
10× TBS, pH 7.5	1×	50 mL
BSA	0.5% (w/v)	2.5 g
Sodium azide, 20%	0.05%	1.25 mL
dd H2O	N/A	446.25 mL
Total		500 mL

Note: Store staining buffer at 4°C. Stable for up to a month.

Blocking buffer		
Reagent	Final concentration	Volume
Staining buffer	N/A	897.5 µL
TruStain FcX (anti-mouse CD16/32)	1.25 µg/mL	2.5 µL
Rabbit serum	10%	100 µL
Total		1 mL

Note: Store the solution at 4°C. Use on the same or next day.

Fixative solution		
Reagent	Final concentration	Volume
RPMI 1640	N/A	8.875 mL
FBS Superior standardized	5%	500 µL
16% formaldehyde, methanol-free	1%	625 µL
Total		10 mL

⚠ CRITICAL: Prepare within 30 min before use.

Dephosphorylation mix with λ phosphatase		
Reagent	Final concentration	Volume
λ protein phosphatase	250 U	0.625 µL
10× NEBuffer for Protein MetalloPhosphatases (PMP)	1×	3 µL
10 mM MnCl2	1 mM	3 µL
dd H2O	N/A	23.375 µL
Total		30 µL / sample

⚠ CRITICAL: Prepare immediately before use.

Control mix without λ phosphatase		
Reagent	Final concentration	Volume
10× NEBuffer for Protein MetalloPhosphatases (PMP)	1×	3 µL
10 mM MnCl2	1 mM	3 µL
dd H2O	N/A	24 µL
Total		30 µL / sample

STEP-BY-STEP METHOD DETAILS

Instant fixation and preparation of single-cell suspensions

Timing: 15 min/mouse + 30 min

The first part of the protocol describes how cells are fixed immediately after organ removal to deactivate kinases and phosphatases while preparing the single cell suspension.

1. Organ extraction and instant cell fixation.
   a. Euthanize mouse by an approved method (e.g., cervical dislocation).
   b. Use surgical scissors and forceps to extract spleen.
   c. Immediately squash the spleen and press through a 100-μm strainer into a Petri dish (ø 50 mm) containing 5 mL of fixative solution, using the plunger of a 2-mL syringe.
   d. Incubate cell suspension for 15 min at room temperature in fixative solution.

⚠ CRITICAL: Euthanasia, removal and processing of tissue should be performed without interruption one mouse at a time. The time between euthanasia and cell fixation should not exceed 5 min.

⚠ CRITICAL: The fixative solution has to be prepared within 30 min before use.

2. Cell filtration and red blood cell lysis.
   a. Pipet cell suspension up and down a few times and filter through a 70 μm nitex mesh into a 15 mL conical tube on ice.
   b. Add 10 mL of RPMI with 5% FBS into each tube.
   c. Centrifuge tubes at 450 × *g* for 5 min at 8°C.
   d. Discard the supernatant, resuspended cell pellet with 2 mL ddH_2O and incubate for 2 min to lyse residual red blood cells.

Alternatives: ACK (Ammonium-Chloride-Potassium) buffer can be used instead of ddH20 to lyse red blood cells.

   e. Stop lysis by adding 10 mL of RPMI with 5% FBS to each tube.
   f. Centrifuge tubes at 450 × *g* for 5 min at 8°C.
   g. Discard the supernatant and resuspend cell pellet in 2 mL RPMI with 5% FBS.
   h. Obtain cell count.

Note: After fixation, all cells are dead. Therefore, all cells must be counted, not live cells.

   i. Adjust the cell concentration to 5×10^7 cells/mL using RPMI with 5% FBS.
   j. Transfer 5×10^6 cells per sample and well to a 96-well u-bottom plate. Use leftover cells for the single-color controls and unstained control.

Note: If the cell population of interest is expected to have a very low frequency more than 5×10^6 cells may need to be stained. If so, antibody dilutions may need to be adjusted to ensure sufficient staining.

Staining procedure and dephosphorylation

Timing: 2-4 h followed by 8-12 h incubation

This section explains how to first stain epitopes of antigens that are methanol-sensitive and afterwards perform methanol permeabilization.

3. Staining of methanol-sensitive surface antigens and methanol permeabilization.
   a. Centrifuge the 96-well plate at 450 × *g* for 3 min at 8°C. Dump supernatants and place the plate on ice.

⚠ CRITICAL: Dump supernatants in a 96-well plate by quickly inverting the plate upside down in a single smooth motion. Tap the plate once on a dry paper towel.

b. Resuspend each well in 50 μL Antibody mix 1 and incubate for 20 min at 4°C protected from light.
c. Add 100 μL TBS (always pH 7.5 in this protocol) to each well and centrifuge the 96-well plate at 450 × *g* for 3 min at 8°C. Dump supernatants and place the plate on ice.
d. Wash cells by resuspending each well with 150 μL TBS, centrifuging the plate at 450 × *g* for 3 min at 8°C and discarding the supernatants.

Note: TBS is used instead of PBS to avoid providing phosphate ions.

e. Resuspend cells in 90 μL of ice-cold methanol (pre-cooled to −20°C) per well and permeabilize for 30 min on ice. Cover the plate to protect from light.

Note: Permeabilization with methanol yields better signal than permeabilization with ethanol, acetone, saponin, or Triton after paraformaldehyde fixation in phosphoflow (Krutzik and Nolan, 2003).

f. Centrifuge the plate at 800 × *g* for 3 min at 8°C. Dump supernatants.

⚠ CRITICAL: From this point on all centrifugation steps must be performed at 800 × *g* to reduce cell loss during washing procedures.

Note: After methanol permeabilization cell pellets are difficult to see in the wells.

g. Wash cells twice by resuspending each well with 150 μL TBS, centrifuging the plate at 800 × *g* for 3 min at 8°C and discarding the supernatants.
h. Resuspend cells in 200 μL TBS.

In this section of the protocol each sample is divided into 2 aliquots, one of which is dephosphorylated. The dephosphorylated sample is later used to quantify the non-specific staining background for phosphoproteins. All samples are stained with the same antibody cocktail that includes antibodies against methanol-stable antigens and phosphoproteins.

4. Dephosphorylation.
   a. Split each sample into 2 samples by transferring 100 μL of each well to a new 96-well u-bottom plate.
   b. Label one plate as "native" and the other plate as "dephosphorylated".
   c. Prepare Dephosphorylation mix with λ phosphatase and Control mix without λ phosphatase.

Note: We use λ protein phosphatase because it eliminates both serine/threonine and tyrosine phosphorylation. We have also tested calf intestinal phosphatase. However, in our experimental setting, it was less effective than λ-protein phosphatase in removing phosphate residues. Calf intestinal phosphatase also has a higher reactivity toward phosphotyrosine residues than phosphoserine/-threonine residues, which makes it less suitable for measuring, for example, phospho-IRF-3 (Ser396).

   d. Centrifuge plates at 800 × *g* for 3 min at 8°C and discard supernatants.
   e. Resuspend each well of the "dephosphorylated" plate with 30 μL of the Dephosphorylation mix with λ phosphatase and each well of the "native" plate with 30 μL of the Control mix without λ phosphatase.
   f. Cover plates with an adhesive plate seal to avoid evaporation. Place the plates in an incubator at 37°C for 30 min.
   g. Add 150 μL staining buffer to each well. Centrifuge plates at 800 × *g* for 3 min at 8°C and discard supernatants.

5. Staining of methanol-stable antigens and phosphoproteins.
   a. Resuspend cells in 25 μL blocking buffer. Block for 10 min at 4°C protected from light.

   ⚠ CRITICAL: The serum used for blocking has to match the host species of the respective anti-phosphoprotein antibodies if these are directly fluorescently labeled.

   b. Add 25 μL of Antibody mix 2 to each sample.
   c. Incubate plates overnight at 4°C protected from light.

   ⏸ **Pause point:** 8–12 h incubation.

   d. Add 100 μL TBS to each well, centrifuge the plate at 800 × *g* for 3 min at 8°C and dump the supernatants.
   e. Wash cells twice by resuspending each well with 150 μL TBS, centrifuging the plate at 800 × *g* for 3 min at 8°C and discarding the supernatants.
   f. Resuspend each well in a 100 μL staining buffer. The samples are ready to be run on a flow cytometer.

   Note: It is advisable to analyze samples on the same day.

Data acquisition and analysis

⏱ **Timing: 1–3 h**

This part of the protocol shows how the data is acquired, corrected for background staining, and finally normalized.

6. Startup flow cytometer and set PMT voltages using single color control and unstained control samples.
7. Acquire 5 × 10^5 events for each sample.

   Note: In case of very rare cell subsets it can be necessary to acquire more events.

8. Analyze data in FlowJo.
   a. After compensation, gate on the cell population of interest and obtain the mean fluorescence intensity (MFI) for the phosphoproteins.
   b. Subtract the MFI of the dephosphorylated sample from the MFI of the corresponding native sample to obtain the ΔMFI. The ΔMFI corresponds to the true signal after background subtraction.
   c. Calculate individual fold change values as $\Delta MFI_i = (MFI_{i,\ native} - MFI_{i,\ dephosphorylated})$ divided by the average of ΔMFI_i values obtained from the reference group in the experiment.
   d. Plot fold-change values in an analysis tool of your choice.

   Note: Apply the same gating strategy to all samples (Figure 1).

EXPECTED OUTCOMES

Phosphoflow is a very reliable method and is in excellent agreement with Western blotting of phosphoproteins (Krutzik and Nolan, 2003). A major advantage of the phosphoflow method over Western blot analysis is the ability to easily interrogate cell subpopulations in primary cell suspensions. Western blot analysis would require lengthy sorting that might alter the phosphorylation state. If the cell subset of interest is rare, as is the case with dendritic cells, for example, it might even be impossible to obtain a sufficient number of cells for a Western blot. The method described here has been

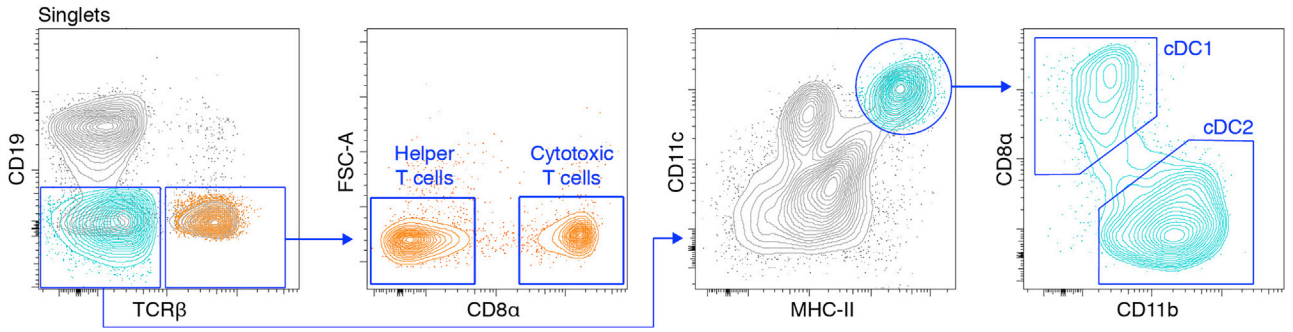

Figure 1. Gating strategy to identify relevant immune cell subsets by flow cytometry

Gating of cytotoxic (TCRβ$^+$CD8α$^+$) and helper (TCRβ$^+$CD8α$^-$) T cells, classical type 1 (CD11c$^+$MHC-II$^+$CD8α$^+$CD11b$^-$) and type 2 (CD11c$^+$MHC-II$^+$CD8α$^-$CD11b$^+$) dendritic cells after removal of duplicates.

successfully applied to spleen and kidney tissue (Khalil et al., 2012; Teichmann et al., 2015; Jütte et al., 2021).

It is important to emphasize that basal phosphorylation is usually relatively weak (Figure 2) compared to induced phosphorylation after treatment of cells with specific agonists. However, this does not mean that it is biologically unimportant. When assessing biological relevance, the duration for which a signaling pathway is activated must be taken into consideration. To allow quantification of native phosphorylation levels, the protocol uses two strategies: 1) instant fixation and 2) background subtraction using matched dephosphorylated samples.

Instant fixation freezes the endogenous phosphorylation state by inactivating phosphatases and kinases. In the presented protocol, it is achieved by immediately crushing the tissue in 1% formalin solution through a strainer after harvesting.

Background signal in flow cytometry results from several factors such as autofluorescence, spectral overlap and non-specific antibody binding. Autofluorescence and spectral overlap can usually be discerned using unstained and fluorescence-minus-one (FMO) controls. Background caused by nonspecific antibody binding is more difficult to identify. Isotope controls are often used for this purpose, but the limitations of their use are well documented (Maecker and Trotter, 2006; Hulspas et al., 2009). In particular, individual isotype-antibody conjugates exhibit varying degrees of background staining depending on their specificity, aggregation, and fluorophore-to-antibody ratio. Biological comparison controls are preferable for the quantification of phosphoproteins. In this protocol, each sample is divided into two subsamples after permeabilization. One subsample is dephosphorylated with λ-phosphatase, and the other is processed without λ-phosphatase but otherwise in exactly the same manner. Detection of the target phosphoproteins is performed with the same antibodies in all samples, so it is not necessary to match the background of the detection antibodies to control antibodies. The MFI of the dephosphorylated sample is then subtracted from the MFI of the corresponding native sample to obtain the true signal (ΔMFI).

LIMITATIONS

Because cells are immediately fixed with formalin, it is not possible to assess viability of the cells using a live/dead stain. However, debris and dead cells can still be excluded using forward and side scatter parameters.

ΔMFI values measured in different experiments can vary considerably. Therefore, it is usually advisable to normalize the data to the reference group in the experiment and plot fold changes to make experiments more comparable.

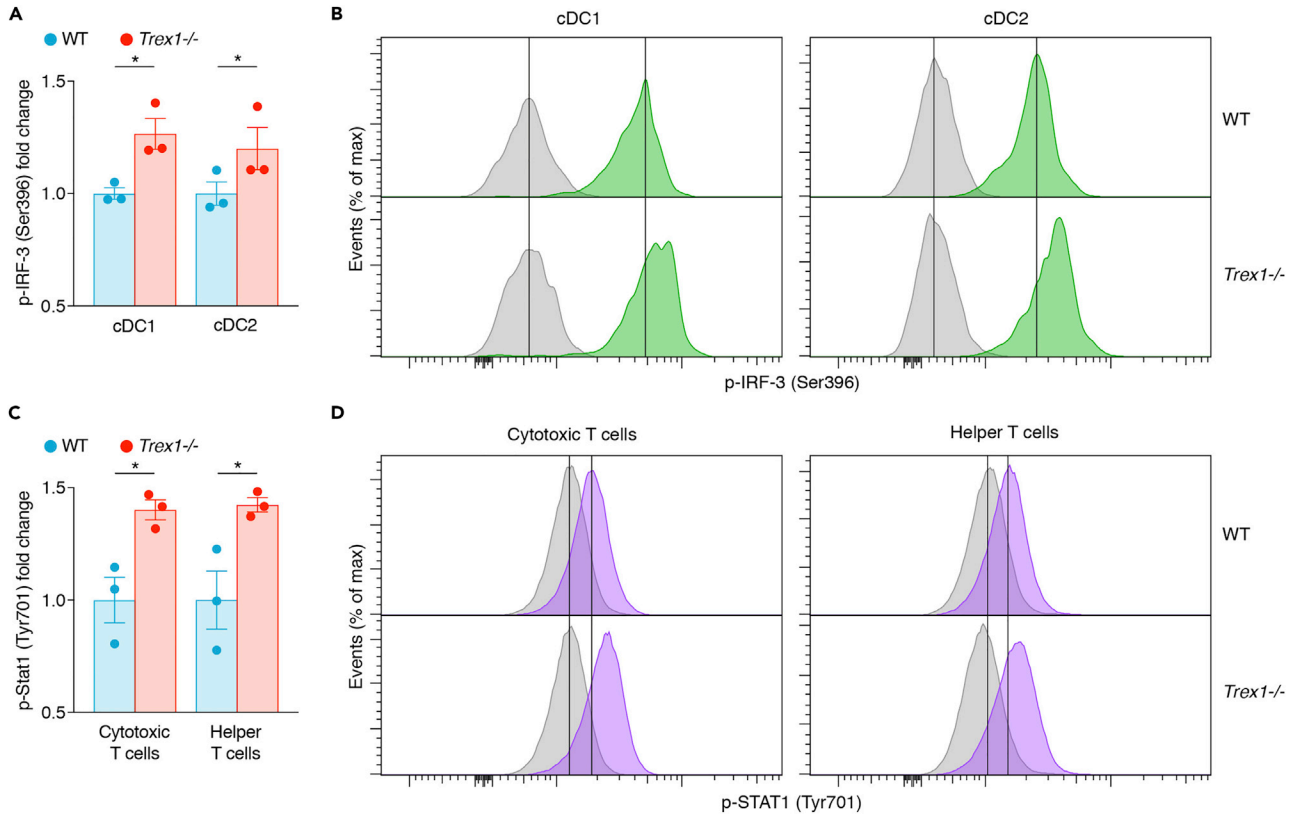

Figure 2. Quantification of p-IRF-3 and p-Stat1 in immune cell subsets

(A) Increased basal IRF-3 signaling activity in splenic cDCs from *Trex1*$^{-/-}$ mice, which develop chronic autoimmunity caused by the recognition of unmetabolized cytosolic DNA by cGAS, compared to those from wild type mice. Fold change of p-IRF-3 mean fluorescence intensity (MFI) of cDCs in instantly fixed spleens from wild type mice (n = 3) and*Trex1*$^{-/-}$ mice (n = 3).

(B) Histograms display representative results for p-IRF-3 (Ser396) of gated cDCs in instantly fixed spleens. Each histogram shows an overlay of dephosphorylated (gray shaded) and native (colored) cells.

(C and D) As in (A) and (B) but for p-Stat1 (Tyr701) in T cells. Data in bar graphs are represented as mean ± SEM. Individual fold change values were calculated as $\Delta MFI_i = (MFI_{i,\ native} - MFI_{i,\ dephosphorylated})$ divided by the average of ΔMFI_i values obtained from wild type mice. Statistically significant differences were determined by two-tailed t-test (*$p < 0.05$).

TROUBLESHOOTING

Problem 1

A large fraction of cells is lost during the procedure (step 3).

Potential solution

After spinning down the cells in the 96-well plate, pour off the supernatant in a single uniform motion and do not invert the plate a second time. Check if the centrifugation steps after methanol fixation are performed at 800 × *g*.

Problem 2

The staining of surface antigens by antibody mix 1 is weak (step 3).

Potential solution

Verify that the antibodies used in mix 1 are coupled to methanol-stable fluorophores.

Problem 3

No dephosphorylation is observed in the dephosphorylated samples (step 4).

Potential solution

This problem can occur if the phosphatase was stored incorrectly or beyond expiry date. Use a fresh batch of phosphatase.

Problem 4

The staining of surface antigens by antibody mix 2 is weak (step 5).

Potential solution

Perform a test experiment to assess whether the epitope of the respective antibody is denatured by methanol permeabilization. If the epitope is compromised by methanol, stain the target antigen before the methanol permeabilization step.

Problem 5

No signal is detected for certain anti-phosphoprotein antibodies (steps 6 and 7).

Potential solution

This problem can be due to a variety of reasons. If possible, run positive control cells with a known high content of the phosphoprotein of interest. Use anti-phosphoprotein antibodies coupled to bright fluorophores, e.g., R-phycoerythrin or Alexa Fluor 647, or secondary antibodies with bright fluorophores when using non-conjugated anti-phosphoprotein antibodies. Check that the photomultiplier (PMT) voltages on the flow cytometer are set correctly.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lino L. Teichmann (lino.teichmann@uni-bonn.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique data sets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

C.K. and L.L.T. designed the experiments, analyzed the data, and wrote the manuscript. C.K., K.C. and R-M.K. performed the experiments. H.L. and A.R-W. provided feedback on and edited the manuscript. P.B. provided resources. L.L.T. supervised the study. All authors had the opportunity to discuss the results and comment on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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