



Protein Phosphatase 1 α and Cofilin Regulate Nuclear Translocation of NF- κ B and Promote Expression of the Anti-Inflammatory Cytokine Interleukin-10 by T Cells

Guido H. Wabnitz,^a Henning Kirchgessner,^a Beate Jahraus,^a Ludmila Umansky,^{a,b} Shirish Shenolikar,^c Yvonne Samstag^a

^aInstitute of Immunology, Ruprecht Karls University, Heidelberg, Germany

^bDepartment of Translational Immunology, German Cancer Research Center, Heidelberg, Germany

^cDuke-NUS Medical School Singapore, Singapore, Singapore

ABSTRACT While several protein serine/threonine kinases control cytokine production by T cells, the roles of serine/threonine phosphatases are largely unexplored. Here, we analyzed the involvement of protein phosphatase 1 α (PP1 α) in cytokine synthesis following costimulation of primary human T cells. Small interfering RNA (siRNA)-mediated knockdown of PP1 α (PP1^{KD}) or expression of a dominant negative PP1 α (D95N-PP1) drastically diminished interleukin-10 (IL-10) production. Focusing on a key transcriptional activator of human IL-10, we demonstrate that nuclear translocation of NF- κ B was significantly inhibited in PP1^{KD} or D95N-PP1 cells. Interestingly, knockdown of cofilin, a known substrate of PP1 containing a nuclear localization signal, also prevented nuclear accumulation of NF- κ B. Expression of a constitutively active nonphosphorylatable S3A-cofilin in D95N-PP1 cells restored nuclear translocation of NF- κ B and IL-10 expression. Subpopulation analysis revealed that defective nuclear translocation of NF- κ B was most prominent in CD4⁺ CD45RA⁻ CXCR3⁻ T cells that included IL-10-producing T_H2 cells. Together these findings reveal novel functions for PP1 α and its substrate cofilin in T cells namely the regulation of the nuclear translocation of NF- κ B and promotion of IL-10 production. These data suggest that stimulation of PP1 α could limit the overwhelming immune responses seen in chronic inflammatory diseases.

KEYWORDS phosphatase, T-cell activation, cytokines

Upon activation, T cells produce a set of cytokines to control innate and adaptive immune responses. The composition of cytokines released by T cells decides whether a pro- or anti-inflammatory immune response prevails. Interleukin-10 (IL-10) is one of the most important cytokines exerting anti-inflammatory effects. This cytokine can be produced by a variety of leukocytes, including monocytes, Th2 cells, and dendritic cells. While NF- κ B represents the major transcription factor that binds the IL-10 promoter and controls IL-10 expression in dendritic cells, the data on regulation of IL-10 expression in T cells are largely inconsistent. Extracellular signal-regulated kinase (ERK) represents an important signaling molecule upstream of IL-10 expression in T cells (1). Furthermore, NF- κ B-dependent expression of GATA-3 is also crucial for the genetic imprinting of the IL-10 locus (2–4). Since the promoter of proinflammatory genes, like gamma interferon (IFN- γ), also contains an NF- κ B binding site (5), activation of NF- κ B can lead to transcription of both pro- and anti-inflammatory genes (1, 6). Thus, the mode of an immune response relies not only on the activation of transcription factors but also on which signaling-module transcription factors are activated.

Signaling modules are often controlled via the reversible phosphorylation of serine, threonine, or tyrosine residues by kinases. Although dephosphorylation by tyrosine phosphatases and the serine/threonine phosphatase (PSTP) PP2B (calcineurin [CaN]) in

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Address correspondence to Guido H. Wabnitz, guido.wabnitz@immu.uni-heidelberg.de.

T cells has been intensely investigated (7, 8), much less is known about the relevance of other PSTPs in T cells. PP1 and PP2A are the major PSTPs in mammalian cells (9, 10). PP2A negatively regulates T-cell activation via dephosphorylation of Carma-1 upstream of NF- κ B (11, 12). PP1 is involved in cell division (13–15) and protein synthesis (16, 17). In tumor cells, inhibition of PP1 suppresses tumor necrosis factor alpha (TNF- α)-induced NF- κ B activation (18, 19). We showed that in T cells PP1 associates with and dephosphorylates the actin-remodeling protein cofilin and that the PSTP inhibitor okadaic acid, which inhibits both PP1 and PP2A, decreases cofilin dephosphorylation (20, 21). Cofilin function is in turn important for immune synapse formation and for cytokine expression in T cells (22). Moreover, PP1 represents a FoxP3 phosphatase in regulatory T cells of rheumatoid arthritis patients (23) and in lymphoma cells (Jurkat cells), and PP1 dephosphorylates AKT during sphingosine-induced apoptosis (24).

Three isoforms of the catalytic subunit of PP1 in mammalian cells are designated PP1 alpha (PP1 α), PP1 beta (PP1 β , also named PP1 δ), and PP1 gamma (PP1 γ), which exists in two splice variants (reviewed in reference 25). PP1 β was reported to control IL-15 and IL-13 expression following incubation of an IL-2 receptor $\alpha\gamma$ -expressing T-cell hybridoma line with IL-2 (26). PP1 α regulates the phosphorylation of BAD upon IL-2 deprivation in murine T-cell lines (27). The relevance of PP1 α for signaling pathways regulating cytokine production by untransformed human T cells is currently unknown.

We show here that a PP1 α knockdown in primary human T cells results in an altered costimulation-induced cytokine profile with decreased IL-10 and IL-2 expression and increased IL-17 expression compared to that in control T cells. We further identified cofilin-dependent nuclear import of NF- κ B as a process downstream of PP1 α as pivotal for this immunomodulation. The NF- κ B pathway is used mainly by CD4⁺ CD45RA⁻ CXCR3⁻ T cells, which contain IL-10-producing T_H2 cells. Thus, our studies have uncovered a novel PP1 α /cofilin/NF- κ B signaling pathway important for (T_H2-type) T-cell mediated anti-inflammatory immune responses.

RESULTS

Inhibition of PP1 α by siRNA-mediated knockdown or expression of a dominant negative PP1 α mutant blocks IL-10 production and promotes IL-17. PP1 α is expressed in primary human T cells (Fig. 1A). To analyze the relevance of PP1 α for T-cell activation, we knocked down PP1 α using small interfering RNA (siRNA). We analyzed different siRNA sequences and identified one that stably knocked down PP1 α expression over a time period of 4 days compared to PP1 α expression in nontargeting control siRNA-treated primary human T cells (Fig. 1A; see Fig. S1 in the supplemental material). Such PP1 α -deficient primary human T cells are referred to as PP1^{KD} cells. The viability of these PP1^{KD} cells was unaltered compared to that of control siRNA-treated cells independent of whether the cells were unstimulated (IgG) or costimulated via cross-linked antibodies against CD3 and CD28 (CD3xCD28) (Fig. 1B).

The concentrations of 19 cytokines and chemokines in the supernatants of PP1^{KD} cells and control siRNA-transfected cells were quantified following costimulation (CD3xCD28) for 24 h. The relative amounts of the analyzed cytokines and chemokines in PP1^{KD} cells compared to those in control siRNA-treated cells are shown in Fig. 1C (the original data are shown in Table 1). The production of IL-1RA, IL-2, IL-5, IL-9, and IL-10 was decreased by at least 33%, and the production of IL-17 was increased by more than 33% (Fig. 1C). The strongest effect was observed for IL-10 production. Compared to that in control cells, the mean IL-10 production after T-cell costimulation was diminished by 1,429 pg/ml, which corresponds to a reduction of 85% \pm 5%.

Since the most profound effects of the PP1 α knockdown were observed for IL-10 production, we investigated the relevance of PP1 α for IL-10 production by an siRNA-independent approach. We took advantage of a green fluorescent protein (GFP)-tagged dominant negative, inactive mutant of PP1 α (D95N-PP1) (28, 29), transiently expressed in primary human T cells. These D95N-PP1 cells, as well as T cells expressing wild-type PP1 α (wt-PP1 α) or the vector alone as a control, were costimulated (CD3xCD28) for 72 h, and IL-10 expression was analyzed by intracellular staining followed by flow cytometry.

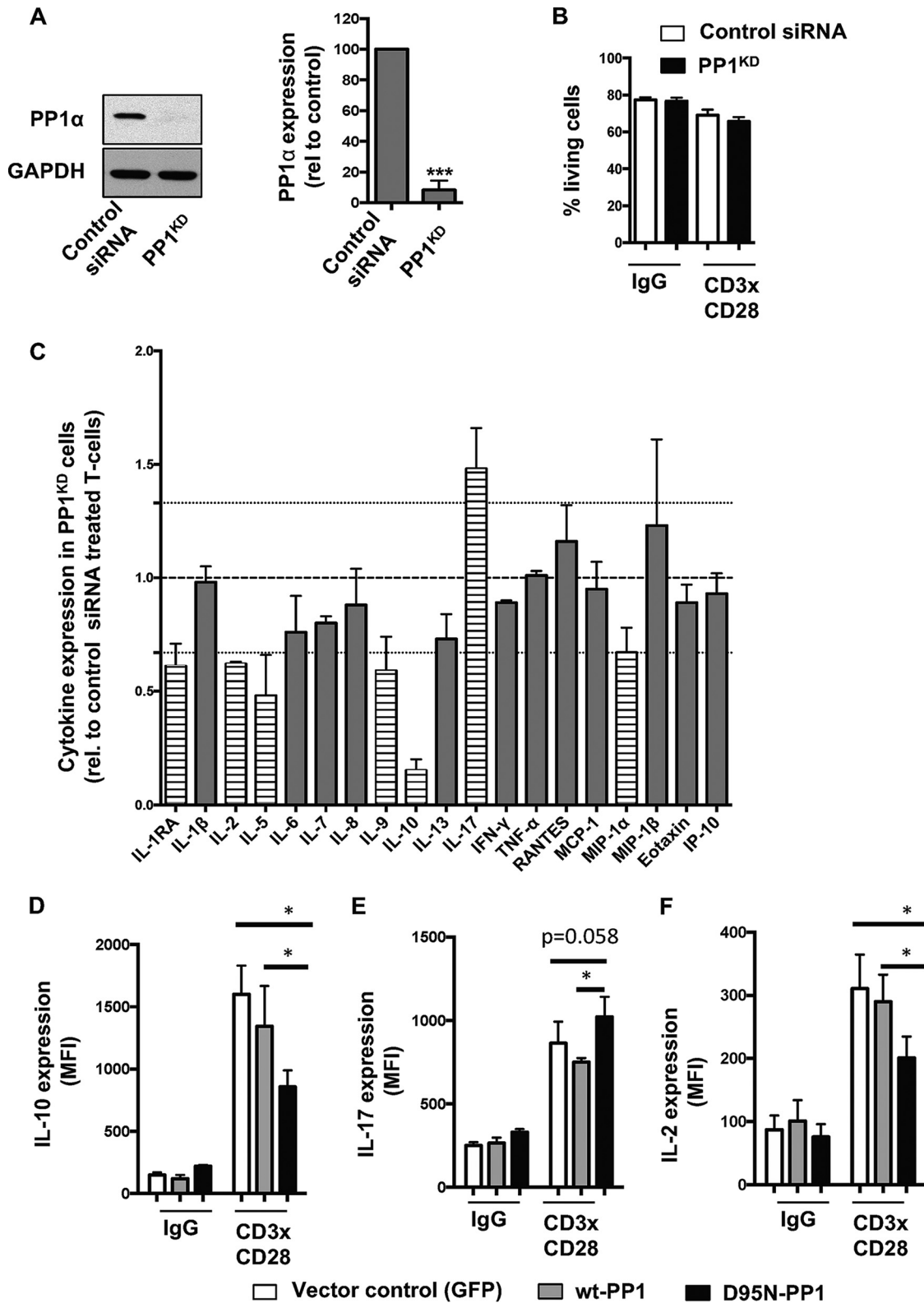


FIG 1 Altered cytokine profile in costimulated PP1^{KD} cells. (A) PP1 α -specific (PP1^{KD}) or nontargeting control siRNAs were transfected into primary human T cells. PP1 α expression was then analyzed in Western blots. Equal loading was confirmed by staining for GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Quantification from three independent experiments is shown on the right, in which the PP1 α expression of control cells was set as 100% ($n = 3$; mean \pm standard error [SE]; ***, $P < 0.001$). (B) Control siRNA-treated T cells or PP1^{KD} cells were stimulated via cross-linked antibodies versus CD3 plus CD28 (CD3xCD28) or settled on IgG control antibodies (IgG). The viability of control or PP1^{KD} cells was analyzed using 7-aminoactinomycin D (7-AAD) labeling and flow cytometry. Shown is the mean percentage of living cells after 72 h ($n = 3$; mean \pm SE). (C) Control or PP1^{KD} T cells were either settled on isotype control

(Continued on next page)

TABLE 1 Concentrations of 19 cytokines and chemokines in the supernatants of PP1^{KD} cells and control siRNA-transfected cells^a

Cytokine or chemokine	No. of repetitions	Mean concn (pg/ml) in:		Relative expression	SE	P value
		Control cells	PP1 ^{KD} cells			
IL-1RA	3	761.4	444.3	0.61	0.10	0.04
IL-1 β	3	27.4	26.8	0.98	0.07	0.74
IL-2	3	15,170	9,349	0.62	0.01	0.0004
IL-5	3	3,051.2	2,238.1	0.48	0.18	0.03
IL-6	3	71.5	49.4	0.76	0.16	0.29
IL-7	3	4.5	3.6	0.80	0.03	0.07
IL-8	3	790.7	714.0	0.88	0.16	0.50
IL-9	3	587.1	328.1	0.59	0.15	0.20
IL-10	3	1,630.5	201.4	0.15	0.05	0.02
IL-13	3	3,388.3	2,701.3	0.73	0.11	0.14
IL-17	3	513.4	751.4	1.48	0.18	0.02
IFN-γ	3	8,186.1	7,368.0	0.89	0.01	0.03
TNF- α	2	28,199.1	28,099.3	1.01	0.02	0.42
RANTES	3	2,764.1	3,404.2	1.16	0.16	0.29
MCP-1	3	16.3	16.1	0.95	0.12	0.96
MIP-1 α	3	5,737.0	3,544.0	0.67	0.11	0.24
MIP-1 β	3	5,980.5	7,442.33	1.23	0.38	0.54
Eotaxin	3	56.97	51.03	0.89	0.08	0.32
IP-10	3	708.7	632.8	0.93	0.09	0.29

^aData for statistically significant results are in bold.

etry. Compared to that in control vector-transfected cells (GFP) or wt-PP1 expressing cells, the amount of intracellular IL-10 was significantly lower following the expression of D95N-PP1 (Fig. 1D). This independently confirmed the importance of PP1 α activity for IL-10 production. Similarly, the increased expression of IL-17 (Fig. 1E) and the decreased expression of IL-2 (Fig. 1F) in PP1^{KD} cells was confirmed in the D95N-PP1 cells. These data showed that PP1 α activity promoted IL-10 and IL-2 production, while inhibiting IL-17 levels.

Inhibition of PP1 α interferes with NF- κ B activation. We next analyzed the potential role of PP1 α in the activation of transcription factors involved in cytokine expression. It was previously shown that okadaic acid, an inhibitor of PP1 and PP2A, provoked NF- κ B phosphorylation in MG63 human osteosarcoma cells, but the specific contribution of PP1 α was not explored (30). In our study, costimulation-related NF- κ B activity was determined by measuring the phosphorylation (Ser529) of the p65 subunit and its translocation to the nucleus by imaging flow cytometry (31–33). While costimulation induced the phosphorylation of NF- κ B (p65) in control siRNA-treated cells, this phosphorylation was completely inhibited in the PP1^{KD} cells (Fig. 2A). Concomitantly, the nuclear translocation of NF- κ B was inhibited in the PP1^{KD} cells. The ImageStream analysis depicted in Fig. 2B (upper panels) shows the typical nuclear translocation of NF- κ B in control siRNA-treated T cells following costimulation (CD3xCD28). While NF- κ B was mainly cytoplasmic in unstimulated control siRNA-treated cells (Fig. 2B, control IgG), there was colocalization of NF- κ B and DAPI (4',6'-diamidino-2-phenylindole) (nuclear stain) in the costimulated T cells (Fig. 2B, control CD3xCD28). Compared to that in costimulated control siRNA-treated cells, the amount of nuclear NF- κ B was significantly lower in the costimulated PP1^{KD} cells (Fig. 2B, PP1^{KD} CD3xCD28).

Other transcription factors that are phosphorylated/activated after T-cell stimulation are the cAMP-response element-binding protein (CREB) and AP-1 (c-Fos/c-Jun). PP1 is

FIG 1 Legend (Continued)

antibodies or costimulated via CD3xCD28 for 24 h. Thereafter, supernatants were collected, and production of cytokines and chemokines was analyzed by multiplex technology. Shown are the amounts of cytokines and chemokines in the supernatant of costimulated PP1^{KD} cells relative to the amount in the supernatant of control siRNA treated cells ($n = 3$, mean \pm SE). The dashed line marks the reference value (costimulated control siRNA-treated T cells), and the dotted lines indicate the $\pm 33.3\%$ expression threshold. In addition, changes of more than 33.3% on expression are marked with hatched columns. (D to F) T cells were transfected with GFP (vector control), GFP-tagged wild-type PP1 α (wt-PP1), or GFP-tagged dominant negative PP1 α (D95N-PP1), respectively. These cells were costimulated (CD3xCD28) for 3 days, and the intracellular IL-10 (D), IL-17 (E), or IL-2 (F) amount (mean fluorescence intensity [MFI]) in GFP-positive cells was analyzed by flow cytometry ($n = 3$; mean \pm SE; *, $P < 0.05$).

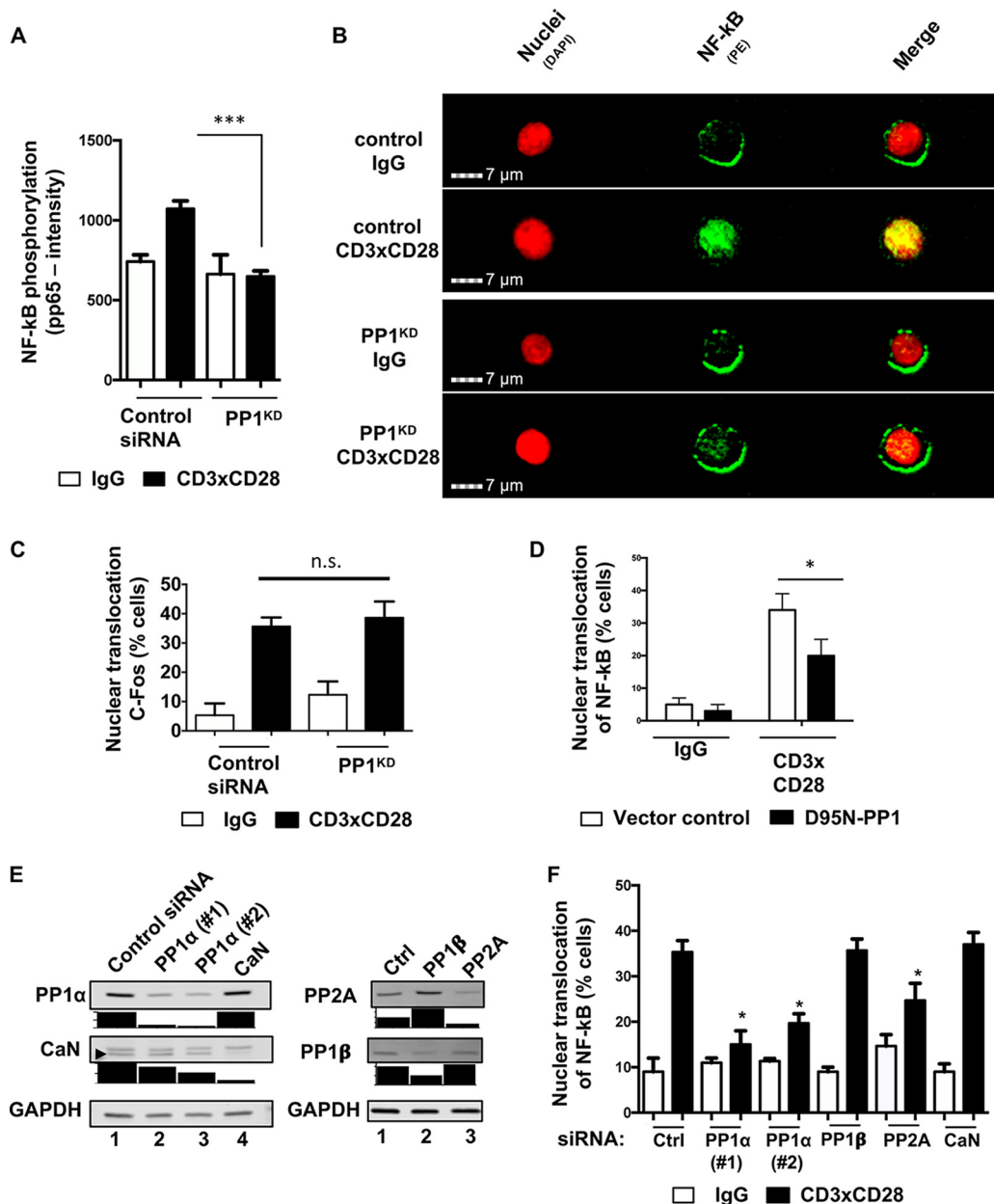


FIG 2 PP1 α regulates activation and nuclear translocation of NF- κ B. (A) Control siRNA-treated T cells or PP1^{KD} cells were costimulated (CD3xCD28) for 30 min. The phosphorylation of NF- κ B (p65) on Ser529 was analyzed using a phosphospecific antibody and imaging flow cytometry ($n = 3$; mean \pm SE; ***, $P < 0.001$). (B) Control siRNA-treated T cells (upper panels) or PP1^{KD} cells (lower panels) were stimulated as described above. Cells were then fixed and stained for nuclei (red) and NF- κ B (p65) (green). Images were acquired using an imaging flow cytometer equipped with a 60 \times objective. Yellow in the overlay (merge) indicates nuclear translocation of NF- κ B. Images are representative of three independent experiments. (C) PP1^{KD} cells (PP1 α siRNA 1) or control siRNA-treated T cells were either costimulated (CD3xCD28) or left unstimulated (IgG). Thereafter, nuclear translocation of c-Fos was quantified using imaging flow cytometry. Shown is the percentage of cells with nuclear c-Fos ($n = 3$; mean \pm SE; n.s., not significant). (D) GFP (vector control) or GFP-tagged dominant negative PP1 α (D95N-PP1) was transfected into T cells. Cells were costimulated via CD3xCD28 or left unstimulated (IgG), and GFP-positive cells were analyzed for nuclear translocation of NF- κ B as described above ($n = 3$; mean \pm SE; *, $P < 0.05$). (E) T cells were treated with control siRNA (Ctrl), with two different siRNAs versus PP1 α , or with siRNAs versus calcineurin (CaN), PP1 β , and PP2A. Protein expression was determined by Western blot analysis (PP1 α and calcineurin) (left blot, CaN [arrowhead]; right blot, PP1 β and PP2A). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) served as a loading control. The black bars under the Western blots indicate the gray value of the respective band. The blots are representative of three independent experiments with similar results. (F) siRNAs were transfected into T cells as indicated, and cells were costimulated via CD3xCD28 antibodies or settled on isotype control antibodies (IgG). Nuclear translocation of NF- κ B was measured by imaging flow cytometry as shown in panel B and depicted as the percentage of cells with nuclear translocation of NF- κ B ($n = 3$; mean \pm SE; *, $P < 0.05$).

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a major CREB phosphatase (34–36). However, in PP1^{KD} T cells, CREB phosphorylation as visualized on Western blots (see Fig. S3 in the supplemental material) was not enhanced. Likewise, the nuclear translocation of c-Fos remained unaltered in PP1^{KD} T cells as analyzed by imaging flow cytometry (Fig. 2C). To apply an siRNA-independent approach, we again made use of the dominant negative PP1 α (D95N-PP1). As observed for the PP1 α knockdown, significantly fewer D95N-PP1-expressing cells than vector control-expressing cells showed nuclear NF- κ B translocation upon CD3 \times CD28 costimulation (Fig. 2D). Together, these results show that PP1 α was important for the activation of NF- κ B, but not for that of CREB or AP-1, in human T cells. Note that while D95N-PP1 was localized in the cytoplasm in unstimulated control cells (see Fig. S2 in the supplemental material, D95N-PP1 IgG), it translocated to the nucleus following costimulation of the T cells through CD3 \times CD28 (Fig. S2, D95N-PP1 CD3 \times CD28).

To strengthen the observation that PP1 α regulates nuclear translocation of NF- κ B, we used another siRNA sequence to knock down PP1 α . In addition, we compared the effects of a knockdown of PP1 β , PP2A, or calcineurin (CaN) to the effects of the PP1 α knockdown. Western blotting confirmed that the knockdown of PP1 α , PP1 β , PP2A, and CaN was efficient (Fig. 2E). Notably, while the knockdown of PP1 α with either PP1 α siRNA 1 or PP1 α siRNA 2 led to a significant reduction of the nuclear translocation of NF- κ B following costimulation (CD3 \times CD28), the knockdown of neither PP1 β nor CaN showed an inhibitory effect on the nuclear translocation of NF- κ B (Fig. 2F). Interestingly, knockdown of PP2A also led to a diminished nuclear translocation of NF- κ B.

PP1 α promotes dephosphorylation of the actin-reorganizing protein cofilin and maturation of the immune synapse. Costimulation induces the okadaic acid-sensitive dephosphorylation of cofilin, which is important for IL-10 and IL-2 expression (21, 37–39). This implicates either PP1 or PP2A, the two major PSTPs, in cofilin regulation. To investigate the role of PP1 α in cofilin dephosphorylation by an independent approach, we costimulated (CD3 \times CD28) PP1^{KD} cells or settled them on isotype control antibodies (IgG) for 30 min. The amount of phosphorylated cofilin was then analyzed using Western blotting (Fig. 3A and B). The knockdown of PP1 α led to a net increase of pCof in primary human T cells as assessed by Western blotting. Importantly, the costimulation (CD3 \times CD28)-induced dephosphorylation of cofilin was also diminished in PP1^{KD} cells compared to control cells. Thus, PP1 α appeared to be a regulator of cofilin dephosphorylation in human T cells.

Cofilin is crucial for the dynamic rearrangement of the actin cytoskeleton and thereby also is involved in the formation of the immune synapse between T cells and their antigen-presenting cells (APCs). To analyze the association between PP1 α function, actin rearrangements, and immune synapse formation, T cells were stimulated via staphylococcal enterotoxin B (SEB)-bearing APCs. After fixation, cells were stained for cofilin, PP1, and CD3. Superresolution microscopy revealed that PP1 α colocalized with cofilin at the immune synapse between T cells and APCs (Fig. 3C). The amount of F-actin in the immune synapse was quantified by imaging flow cytometry as described before (40), and it turned out to be significantly lower in PP1^{KD} cells (Fig. 3D and E). Since actin cytoskeleton rearrangements are crucial for immune synapse formation and maturation, we also analyzed the clustering of LFA-1 (Fig. 3D and F). Indeed, the amount of LFA-1 within the immune synapse (IS), as a sign of IS maturation, was likewise significantly reduced in PP1^{KD} cells compared to control cells. Therefore, our experiments strongly suggest that the regulation of cofilin by PP1 α affects not only nuclear translocation of NF- κ B but also immune synapse formation and maturation.

PP1 α and cofilin colocalize with NF- κ B in the nuclei of stimulated T cells. The data described above demonstrated that PP1 α regulated both nuclear translocation of NF- κ B and cofilin dephosphorylation. Cofilin contains a nuclear localization signal (NLS). While cofilin was mostly absent in the nuclei of unstimulated T cells, it showed a nuclear localization upon T-cell stimulation (38). However, whether nuclear translocation of cofilin played a role in NF- κ B activation was unknown. We therefore determined the localization of cofilin, PP1 α , and NF- κ B in APC-stimulated T cells by superresolution

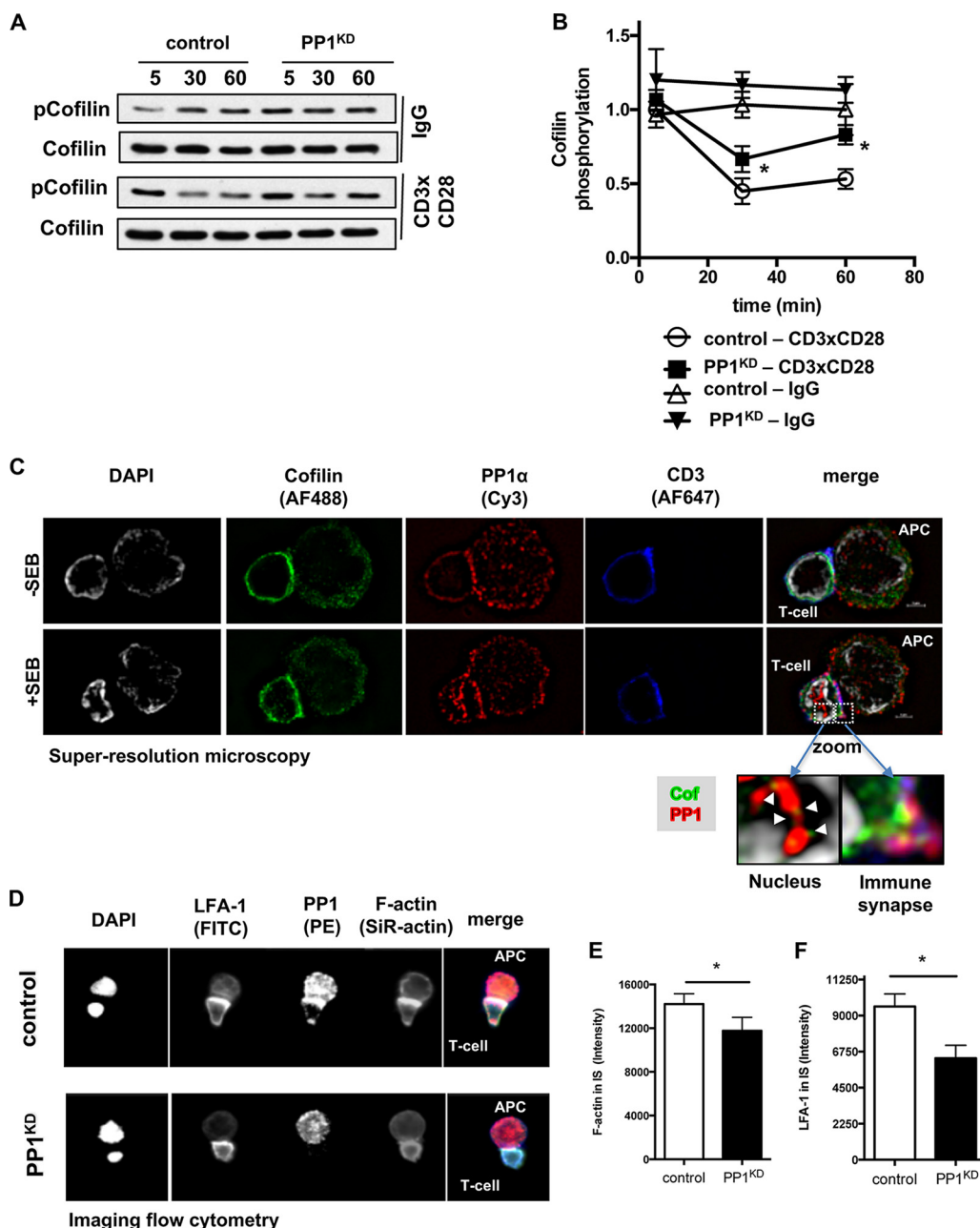


FIG 3 PP1 α promotes dephosphorylation of cofilin and immune synapse maturation in primary human T cells. (A) Control siRNA-treated T cells or PP1^{KD} cells were either left unstimulated (IgG) or costimulated using cross-linked antibodies against CD3 in combination with antibodies against CD28 (CD3xCD28). The phosphorylation state of cofilin was analyzed by Western blotting at the indicated time points using antibodies against phospho-cofilin (pCofilin) and cofilin (Cofilin). (B) A quantification of the phosphorylation state of cofilin is shown as ratio of the gray values of the phosphoprotein to the total protein from the Western blots (lower part) (mean \pm SE; $n = 3$; *, $P < 0.05$). (C) The superresolution microscopy images show the localization of cofilin (green) and PP1 α (red) in T cells that were conjugated to APCs that were either loaded with SEB (+SEB) or left unloaded (-SEB). The enlarged image shows the localization of PP1 α and cofilin in the nucleus or immune synapse, respectively. (D) The localization of LFA-1 was analyzed in control siRNA-treated cells or PP1^{KD} cells using imaging flow cytometry. The figure shows images of the nuclei (DAPI), LFA-1 (CD18-fluorescein isothiocyanate [FITC]), PP1 (PE), and F-actin (SiR-actin) and is representative of three independent experiments. (E and F) Quantification of F-actin (E) and LFA-1 (F) in the immune synapse ($n = 3$; mean \pm SE; *, $P < 0.05$).

microscopy. This allowed us to analyze small (about 120-nm) protein clusters and to resolve perinuclear heterochromatin and distinguish it from euchromatin structures in the nucleus (Fig. 4A). This showed that SEB-stimulation induced a nuclear translocation of cofilin and NF- κ B (Fig. 4A and B). Note that stimulation of T cells with SEB-bearing

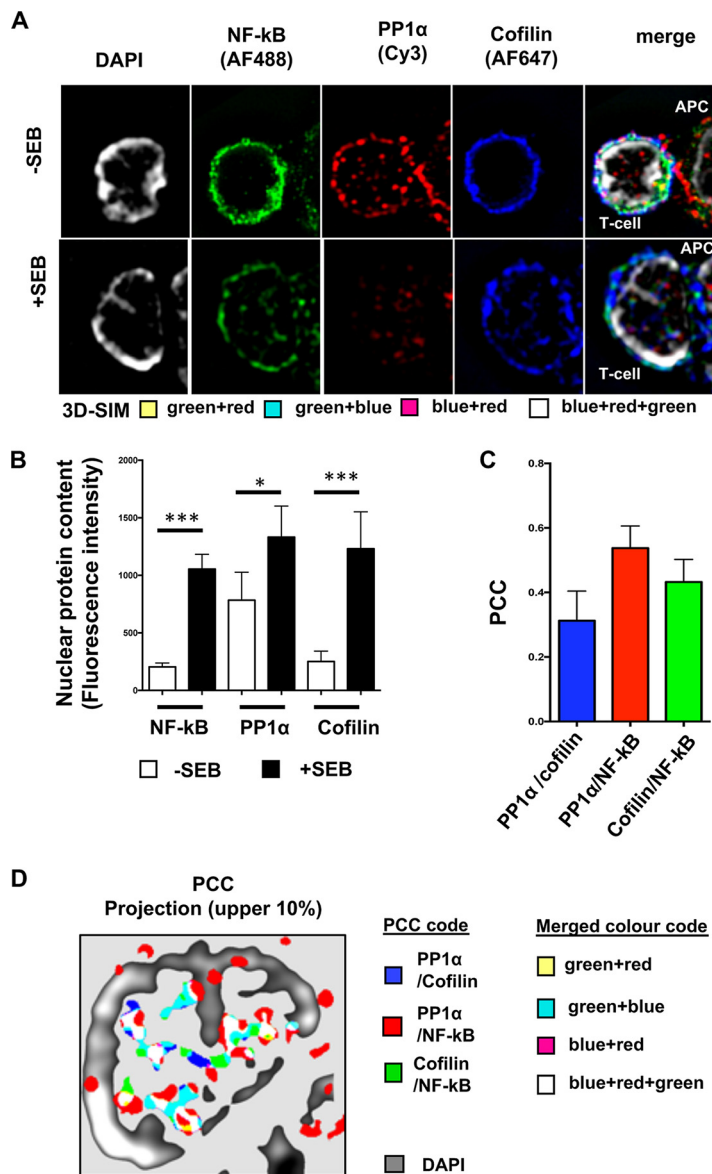


FIG 4 Colocalization of cofilin, PP1α, and NF-κB in the nucleus. (A) T cells were mixed with unloaded APCs (upper row) or stimulated via SEB-bearing APCs (lower row) for 45 min. After fixation, cells were stained for DNA (DAPI, white), NF-κB (green), PP1α (red), and cofilin (blue). Images of cell couples (APCs were cropped out) were acquired using superresolution microscopy (3D-SIM). The images are representative of three independent experiments. (B) The fluorescence intensities for NF-κB, PP1α, or cofilin staining in the nuclei of unstimulated (-SEB) or stimulated (+SEB) T cells were quantified in 25 cells taken from 3 experiments for each condition (mean ± SE; *, $P < 0.05$; ***, $P < 0.001$). (C) Pearson's correlation coefficient (PCC) in the nucleus was calculated for PP1α/cofilin (blue), cofilin/NF-κB (green), or PP1α/NF-κB (red) in 25 cells. (D) ROIs were defined for the upper 10% PCC values and superimposed on an image showing the DAPI signal in gray.

APCs is weaker than antibody-mediated stimulation (CD3xCD28). Accordingly, this mode of activation led to a weaker nuclear translocation of NF-κB. PP1α was constitutively present in the nucleus. However, the SEB-induced increase in nuclear PP1α was still significant.

To analyze the relationship between these proteins within the nucleus, the Pearson correlation coefficient (PCC) was calculated for SEB-stimulated cells. PCC values between 0 and 1 indicate colocalization, and negative values indicate antilocalization.

The PCC values for PP1α/cofilin (0.31 ± 0.15), cofilin/NF-κB (0.53 ± 0.23), and PP1α/NF-κB (0.43 ± 0.19) suggested a colocalization of the respective proteins (Fig. 4C).

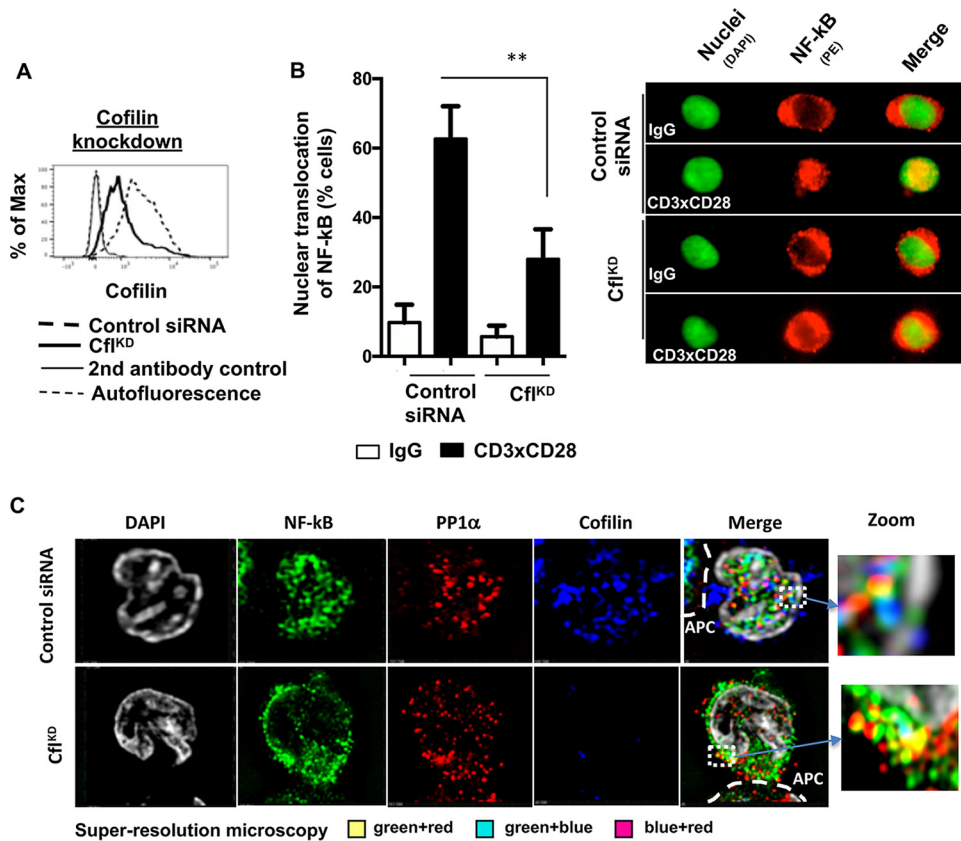


FIG 5 Defective nuclear translocation of NF- κ B in cofilin knockdown T cells. (A) Jurkat T cells were treated with control siRNA or with cofilin-specific siRNA to create cofilin knockdown (Cfl^{KD}) T cells and subsequently analyzed by flow cytometry. The figure shows the autofluorescence and the secondary antibody control staining. The histogram is representative of three experiments. (B) Nuclear translocation of NF- κ B was analyzed in control siRNA-treated T cells or cofilin knockdown (Cfl^{KD}) cells after costimulation by imaging flow cytometry. The experiment was performed as described above. Representative images are shown on the right, and the corresponding quantification is depicted on the left ($n = 3$; mean \pm SE; **, $P < 0.01$). (C) Control or cofilin knockdown (Cfl^{KD}) cells were stimulated via SEE-loaded APCs. After fixation, cells were stained for nuclei (DAPI, white), NF- κ B (green), PP1 α (red), and cofilin (blue). The localization of these proteins was determined by superresolution microscopy (APCs were cropped out). The enlarged image shows the partial colocalization of PP1 α , NF- κ B, and cofilin. The images are representative of 30 cells taken from 3 independent experiments.

To analyze the area of highest colocalization within the nucleus more precisely, we defined regions of interests (ROIs) marking the upper 10% PPC values for PP1 α /cofilin (Fig. 4D, blue), cofilin/NF- κ B (Fig. 4D, green), or PP1 α /NF- κ B (Fig. 4D, red) and superimposed these ROIs on an image showing the structure of the chromatin (Fig. 4D, gray). This superimposition shows that all three proteins were closely associated at euchromatin structures (Fig. 4A and D). This suggested that cofilin may regulate the nuclear translocation of NF- κ B and its association with chromatin.

A PP1 α -cofilin axis regulates NF- κ B activation in costimulated human T cells.

We next determined whether cofilin was required for the nuclear translocation of NF- κ B. To this end, cofilin was knocked down in Jurkat T cells as described before (Fig. 5A). Note that although these cells differ in some aspects from primary human T cells (41), these cells were necessary for these experiments since the knockdown of cofilin in primary human T cells is lethal (42). Following costimulation by CD3xCD28 antibodies (Fig. 5B) or by superantigen (staphylococcal enterotoxin E [SEE])-loaded Raji cells as APCs (Fig. 5C), the localization of NF- κ B (Fig. 5B and C) and cofilin and PP1 α (Fig. 5C) was determined by imaging flow cytometry (Fig. 5B) or superresolution microscopy (Fig. 5C). The cofilin knockdown (Cfl^{KD}) diminished the nuclear translocation of NF- κ B in CD3xCD28 T cells as quantified by imaging flow cytometry (Fig. 5B) and upon superantigen-dependent activation (Fig. 5C, lower panels) (mean intensity of nuclear

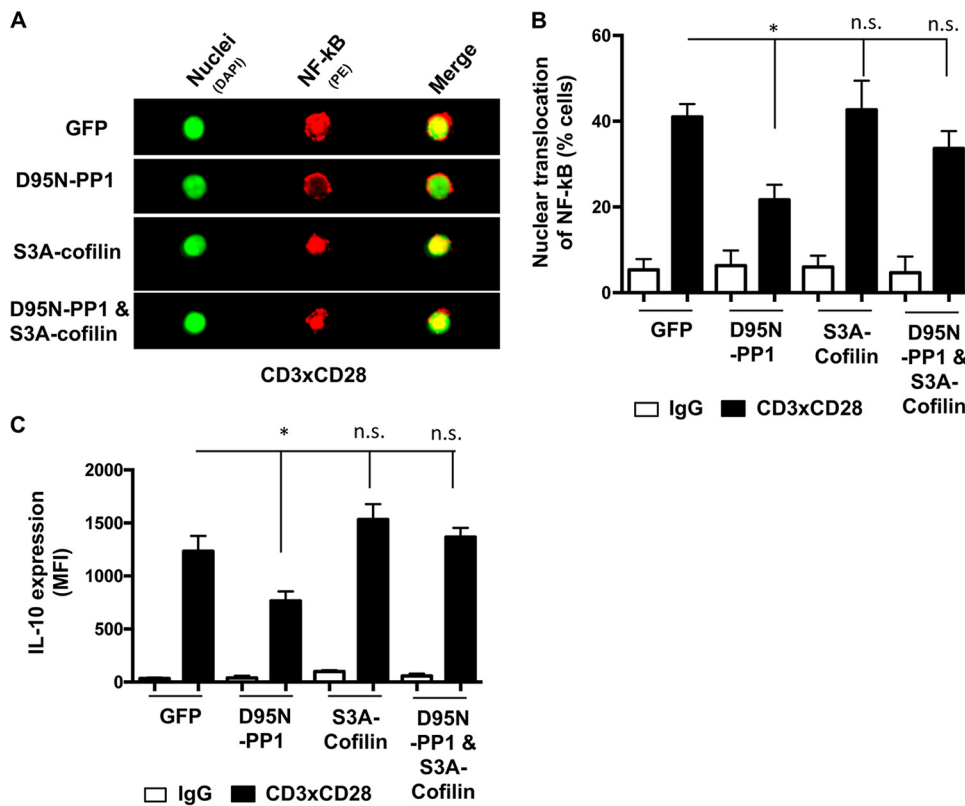


FIG 6 Nonphosphorylatable S3A-cofilin rescues nuclear translocation of NF- κ B and IL-10 production in T cells expressing dominant negative PP1 α . (A and B) GFP (control vector) or GFP-tagged D95N-PP1, S3A-cofilin, or a combination of D95N-PP1 and S3A-cofilin was expressed in primary human T cells. The nuclear translocation of NF- κ B was measured after costimulation using imaging flow cytometry. The images (gated on GFP-positive cells) are representative of three independent experiments, and the quantification shows the percentage of cells with a nuclear translocation of NF- κ B ($n = 3$; mean \pm SE; *, $P < 0.05$; n.s., not significant). (C) T cells expressing either GFP (control vector) or GFP-tagged D95N-PP1, S3A-cofilin, or D95N-PP1 plus S3A-cofilin were costimulated via cross-linked CD3 and CD28 antibodies for 72 h. Thereafter, IL-10 expression was measured by flow cytometry as described above ($n = 3$; mean \pm SE; *, $P < 0.05$; n.s., not significant).

NF- κ B in control siRNA and Cfl^{KD} = $1,456 \pm 145$ and 932 ± 295 , respectively). As described above for primary human T cells, superresolution microscopy revealed that cofilin, PP1 α , and NF- κ B partially colocalized in the nuclei of Jurkat T cells preincubated with control siRNA and activated by superantigen-loaded APCs (Fig. 5C, upper panels). Importantly, while the nuclear translocation of NF- κ B was significantly reduced in Cfl^{KD} cells, PP1 α and NF- κ B partially colocalized in the cytoplasm of these cells (PCC = 0.57 ± 0.22). Thus, cofilin likely regulated the nuclear translocation of NF- κ B through its association with and activation by PP1 α .

These data suggested that the increased phosphorylation and inactivation of cofilin in PP1^{KD} cells were critical for the inhibition of nuclear translocation of NF- κ B. As further support for the existence of a PP1 α -cofilin-NF- κ B signaling module, we expressed the dominant negative PP1 α (D95N-PP1) in either the absence or presence of a constitutively active cofilin, in which the phosphorylation site at serine 3 was mutated to a nonphosphorylatable alanine (S3A-cofilin) in primary human T cells. After costimulation, the nuclear translocation of NF- κ B was measured using imaging flow cytometry (Fig. 6A and B). As observed for PP1^{KD} cells, expression of D95N-PP1 interfered with costimulation-induced nuclear translocation of NF- κ B (Fig. 6B, D95N-PP1). Most importantly, coexpression of constitutively active nonphosphorylatable S3A-cofilin (Fig. 6B, D95N-PP1 and S3A-cofilin) abrogated this effect, resulting in nuclear levels of NF- κ B comparable to those in cells transfected with vector control only (Fig. 6B, GFP). These results confirmed that PP1 α regulates the nuclear translocation of NF- κ B via cofilin

dephosphorylation. Consequently, while expression of D95N-PP1 interfered with IL-10 expression, the simultaneous expression of D95N-PP1 and 3SA-cofilin restored the ability of the cells to produce IL-10 (Fig. 6C). Together, the data showed that PP1 α -mediated cofilin activation was required for nuclear translocation of NF- κ B and differential cytokine production in human T cells.

The PP1 α -NF- κ B pathway is preferentially used by T_H2 cells. The knockdown of PP1 α differentially modulated cytokine and chemokine production (Fig. 1 and Table 1). Thus, while IL-10 production was inhibited in PP1^{KD} cells, other cytokines known to be regulated by NF- κ B, e.g., IFN- γ , were not inhibited in PP1^{KD} cells. One possible explanation for this surprising finding was a potential T-cell subpopulation dependency on PP1 α . CD4⁺ and CD4⁻ T cells expressed similar levels of PP1 α (Fig. 7A). However, the amount of PP1 α was significantly higher in naive CD4⁺ CD45RA⁺ (Fig. 7B) and naive CD4⁻ CD45RA⁺ (Fig. 7C) T cells than in the respective memory T cells, i.e., CD4⁺ CD45RA⁻ or CD4⁻ CD45RA⁻ T cells. Interestingly, costimulation-induced nuclear translocation of NF- κ B was reduced only in CD4⁺ PP1^{KD} cells and not in CD4⁻ PP1^{KD} T cells (Fig. 7D). Within these CD4⁺ T cells, nuclear translocation was inhibited only in CD45RA⁻ (memory) CD4⁺ PP1^{KD} cells and not in CD45RA⁺ (naive) CD4⁺ PP1^{KD} cells (Fig. 7E).

The strongest effect of the PP1 α knockdown on cytokine production was shown for IL-10. This cytokine is produced, e.g., by CXCR3⁻ T_H2 cells but not by CXCR3⁺ T_H1 cells. Remarkably, while CD4⁺ CD45RA⁻ CXCR3⁻ PP1^{KD} cells showed a decrease in nuclear translocation of NF- κ B, CD4⁺ CD45RA⁻ CXCR3⁺ PP1^{KD} showed no significant change in the nuclear translocation of NF- κ B compared to that in control siRNA-treated cells (Fig. 7F). This demonstrated the preferential utilization of the PP1 α -NF- κ B pathway by T_H2 but not T_H1 cells. Cell population-specific PP1 α usage may, at least partially, explain why IL-10 expression is sensitive to the PP1 α knockdown whereas IFN- γ expression is not.

DISCUSSION

The activation and function of T cells are tightly controlled to ensure an intact immune surveillance and adequate immune responses. Identifying molecular targets for immunomodulation requires greater knowledge of the impact of fine-tuning signaling cascades in T cells, many of which involve a reversible protein phosphorylation. We analyzed the function of PP1 α in primary human T cells from healthy donors and found that PP1 α was crucial for differential changes in cytokine expression mediated by a signaling module containing PP1 α , cofilin, and NF- κ B.

Although a number of studies have identified protein kinases involved in NF- κ B activation, fewer studies have focused on phosphatases that regulate either inhibitor κ B (I κ B) or NF- κ B phosphorylation. For example, the PP2C family member WIP1 (43) and PP2A (44) were shown to dephosphorylate the NF- κ B subunit p65 at S536 in nonhematopoietic tumor cells. In human T cells, however, B56 γ , a regulatory subunit of PP2A, was upregulated following prolonged T-cell costimulation and functioned as negative regulator for NF- κ B by dephosphorylation of large I κ B kinase (IKK) (45). PP4R1 was also upregulated following prolonged phytohemagglutinin (PHA) stimulation of untransformed human T cells and assembled a PP4/PP4R1 holoenzyme that negatively regulated IKK and counteracted NF- κ B activation (46). Thus, PP2A and PP4 were shown to inactivate NF- κ B in later phases of T-cell activation. In contrast, our study highlights for the first time an early event (within 30 min) that follows costimulation of human T cells via CD3 and CD28 in which PP1 α , together with its substrate cofilin, promotes the activation and nuclear translocation of NF- κ B, demonstrating a critical role for PP1 α in early T-cell signaling.

Cofilin is an important substrate of PP1 α , and it is dephosphorylated (activated) upon T-cell costimulation. We described earlier that cofilin-actin interactions are important for expression of the T-cell growth factor IL-2 and the T_H2 cytokine IL-10 in T cells (22). The molecular mechanisms of how cofilin impacted this cytokine production, however, were not known. Cofilin contains a nuclear localization signal (NLS), and we found that cofilin is localized in the nucleus upon stimulation with SEB-loaded APCs

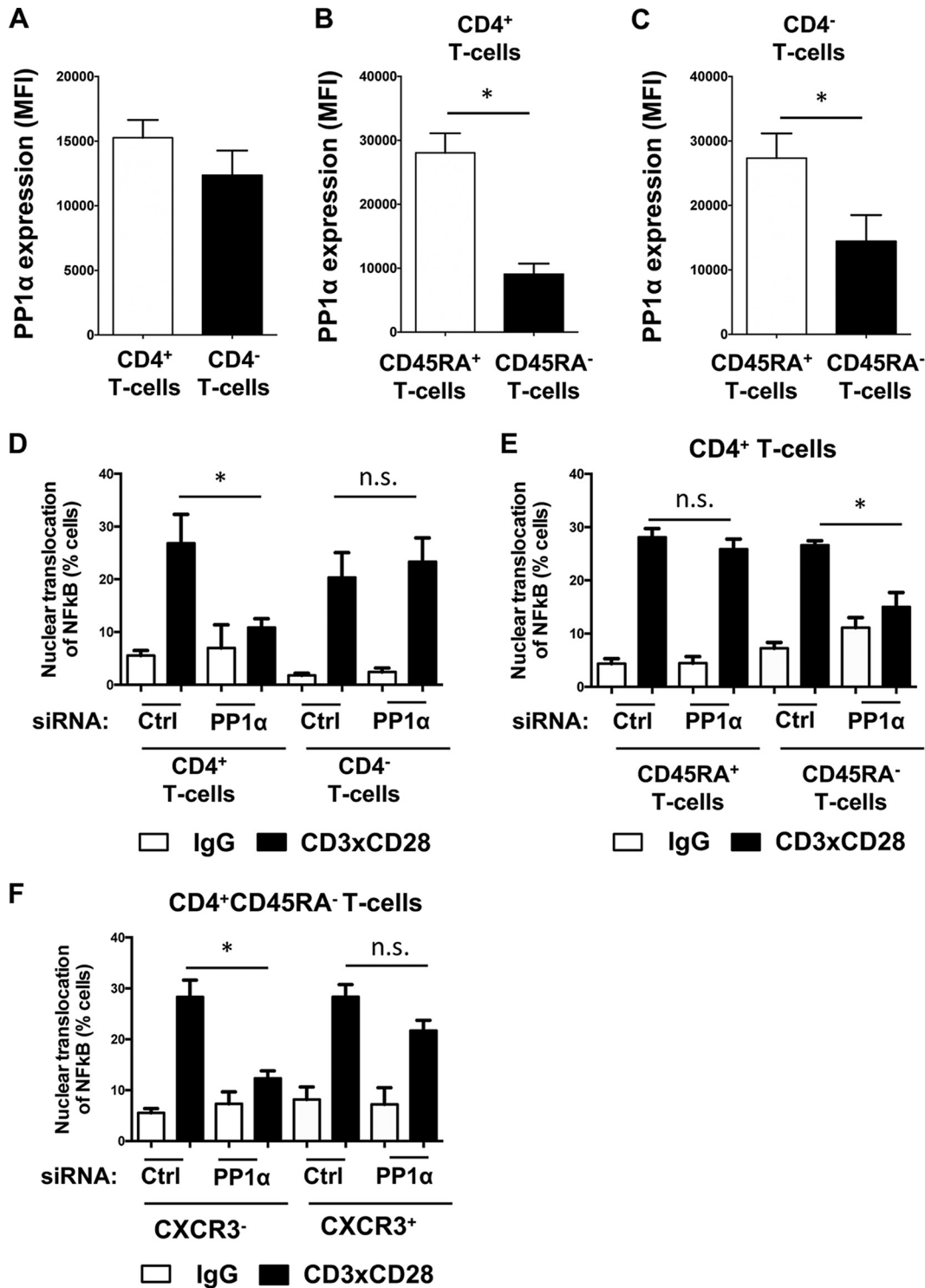


FIG 7 Subpopulation-restricted inhibition of nuclear translocation of NF-κB in PP1^{KD} cells. (A to C) Expression of PP1α in CD4⁺ and CD4⁻ T cells (A), in CD4⁺ CD45RA⁺ or CD4⁺ CD45RA⁻ cells (B), or in CD4⁻ CD45RA⁺ or CD4⁻ CD45RA⁻ T cells (C) was analyzed by flow cytometry. Shown is the mean fluorescence intensity (geometric mean) of the respective T-cell subpopulation (*n* = 3; mean ± SE; n.s., not significant). (D to F) Nuclear translocation of NF-κB was analyzed in control siRNA-treated cells (Ctrl) or PP1α siRNA treated cells (PP1α) using imaging flow cytometry as described above. T-cell subpopulations were gated as CD4⁺ and CD4⁻ (D), CD4⁺ CD45RA⁺ or CD4⁺ CD45RA⁻ (E), or CD4⁺ CD45RA⁻ CXCR3⁺ or CD4⁺ CD45RA⁻ CXCR3⁻ (F) T cells. Shown is the percentage of cells containing nuclear NF-κB (*n* = 3; mean ± SE; *, *P* < 0.05; n.s., not significant).

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(this work) or mitogenic CD2 antibodies (38). Since actin lacks an NLS, we speculated that cofilin shuttles actin to the nucleus, which in turn may regulate cytokine production. Indeed, it was later shown that cofilin controls actin binding to importins (47), which are important regulators for nucleocytoplasmic protein transport (reviewed in reference 48). Here we uncovered another, previously unknown key function of cofilin in gene regulation in T cells, namely, that nuclear translocation of the transcription factor NF- κ B was dependent on cofilin. We took advantage of different experimental systems, i.e., siRNA-mediated knockdown of PP1 α , expression of a dominant negative variant of PP1 α (D95N-PP1), and/or expression of constitutively active cofilin (S3A-cofilin) in primary human T cells. In addition, we knocked down cofilin in the T-lymphoma line Jurkat. The knockdown of cofilin or PP1 α , or expression of the dominant negative D95N-PP1, all attenuated the nuclear shuttling of NF- κ B in response to T-cell costimulation. Notably, knockdown of PP2A, which is an additional cofilin phosphatase (21), also interfered with the nuclear translocation of NF- κ B in short-term costimulated T cells. Most importantly, expression of S3A-cofilin rescued D95N-PP1-dependent inhibition of nuclear translocation of NF- κ B. Together, these findings imply that PP1 α regulated the nuclear translocation of NF- κ B through the dephosphorylation (activation) of cofilin. This novel signaling event likely contributes to the fine-tuning of NF- κ B regulation upon T-cell costimulation. Future work will uncover whether this signaling pathway is also activated by other stimuli (e.g., TNF- α) and whether it is used in both the canonical and noncanonical activation of NF- κ B.

NF- κ B contains a nuclear localization signal (NLS), which is masked by I κ B in unstimulated cells. Upon cell activation, I κ B is phosphorylated by IKK and degraded, and the NLS of NF- κ B is unmasked, leading to the binding of importins and the subsequent nuclear transport of NF- κ B (49, 50). Cofilin (which also contains an NLS) colocalized with NF- κ B in the cytoplasm and in the nucleus. Since cofilin controls actin binding to importins (47), it is tempting to speculate that cofilin facilitates nuclear shuttling of NF- κ B by regulating importin binding. This hypothesis is supported by reports showing that the functionality of importins is required but not necessarily sufficient for nuclear NF- κ B transport; for example, a cytoskeleton-assisted nuclear transport was described (51). Further work will be required to elucidate these mechanisms.

The strongest functional effect of the PP1 α knockdown was the decrease in production of IL-10. IL-10 is an important cytokine that is secreted mainly by monocytes but also by T_H2 cells. It limits immune responses, thereby preventing the overshooting of immune responses and chronic inflammation. Yet, under certain conditions, IL-10 can also lead to enhanced STAT1 signaling after type I interferon priming and thus exert proinflammatory properties (52). Several transcription factors regulate IL-10 expression. These include the signal transducers and activators of transcription (STAT), CREB, CCATT enhancer/binding protein (C/EBP), and NF- κ B (53). Moreover, different transcription factors and upstream signaling pathways (e.g., ERK) and lymphocyte-specific protein tyrosine kinase (LCK) initiate IL-10 transcription, depending on the cell type and type of stimulus (54). IL-10-inducing signaling cascades have been studied extensively in myeloid cells but less thoroughly in primary human T cells (55). Here we show for the first time that nuclear translocation of NF- κ B and IL-10 expression after stimulation of primary human T cells via TCR/CD3 and CD28 are, at least in part, dependent on PP1 α function. Of note, the knockdown of PP1 α in T cells did not influence the serine/threonine phosphorylation of ERK or LCK (see Fig. S4 in the supplemental material), which partially contradicts prior results obtained with the PP1 inhibitor tautomycin (56). We also did not observe an effect of the PP1 α knockdown on c-Fos activation, which is downstream of ERK. This suggests that the PP1 α -cofilin axis is somewhat specific for NF- κ B in T cells.

While CREB, another putative PP1-dependent transcription factor, interacts with PP1 under certain conditions (36), the phosphorylation of CREB was not impacted upon costimulation of PP1^{KD} T cells compared to control cells (see Fig. S3 in the supplemental material). We provide two molecular explanations for this finding. First, nuclear PP2A

can also dephosphorylate CREB, a constitutively nuclear protein with a 30-fold-higher affinity than nuclear PP1. Thus, PP2A may compensate in the absence of PP1 α (57). Second, upon T-cell costimulation, CREB is regulated by nuclear calcium signaling (58). Therefore, CREB seems to be involved in a costimulatory signaling pathway very different from that of PP1 α and cofilin.

Interestingly, although the expression of many cytokines and chemokines is dependent on NF- κ B, we observed differential effects after costimulation of PP1^{KD} cells. Thus, while IL-10 expression was strongly decreased, the amount of IFN- γ , IL-6, or IL-8 was not significantly changed. This observation is likely due to distinct dependencies of the nuclear translocation of NF- κ B on PP1 α in T-cell subpopulations. Thus, we found that nuclear translocation of NF- κ B was more profoundly modulated in memory T-helper cells that were negative for CXCR3. These cells comprise T_H2 cells, which express IL-10. Notably, IFN- γ , IL-6, and IL-8 are produced by CXCR3⁺ T_H1 cells, in which the knock-down of PP1 α did not interfere with nuclear translocation of NF- κ B.

Compared to that in control cells, expression of the proinflammatory cytokine IL-17 was also enhanced upon costimulation of PP1^{KD} T cells. While the IL-10 and IL-2 promoters themselves contain NF- κ B-binding sites (59, 60), IL-17 gene transcription is regulated mainly by the transcription factor retinoic acid receptor-related orphan nuclear receptor gamma (ROR γ t), the expression of which is dependent on NF- κ B. Thus, IL-17 gene expression can be indirectly regulated by NF- κ B (61). In contrast, other reports showed that NF- κ B is dispensable for IL-17 expression (62), and hyperactivation of NF- κ B, e.g., in USP-18-deficient T cells, even interfered with IL-17 expression (63). Given this complexity of IL-17 gene regulation, more work will be needed to understand how PP1 α antagonizes IL-17 production.

In conclusion, since PP1 α promotes production of the anti-inflammatory cytokine IL-10 and impedes an (overshooting) IL-17 response, it may be crucial for ameliorating local inflammation. Such a net anti-inflammatory cytokine milieu mediated by PP1 α could be of special importance in the gut because IL-10 is a major regulator of homeostatic hyporesponsiveness of gut lymphocytes (64, 65) and IL-17 is strongly upregulated during inflammatory bowel disease (66). IL-17 also plays a crucial role in inflammatory skin diseases such as psoriasis (67). The PP1 α -cofilin-NF- κ B module may thus represent an important modulating factor for controlling human inflammatory and homeostatic T-cell-mediated immune responses.

MATERIALS AND METHODS

Cell culture and transfection procedures. Primary human T cells were purified with negative magnetic bead selection using the Pan T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). T-cell blasts were generated by incubation with PHA-L (2 μ g/ml) for 24 h. Thereafter, cells were washed twice by centrifugation (290 \times g, 10 min) and resuspended in culture medium containing 80 U/ml IL-2. For siRNA approaches, T-cell blasts were used at day 7, if the viability was above 90%, using Accell siRNA (PP1 α siRNA 1; GUCUCUUGAAUAAAGGUCA) or control siRNA (Dharmacon, Lafayette, LA). The cells were used for experiments at day two after transfection. The cofilin knockdown was performed as described elsewhere (42).

For cDNA transfection into primary human T cells (GFP or GFP-tagged PP1 constructs; for details, see references 28 and 29), the human T-cell Nucleofector kit (Amaxa Biosystems, Cologne, Germany) was used according to the manufacturer's instructions.

Reagents and antibodies. Stimulating antibodies (20 ng/ml OKT3 [ATCC, Manassas, VA] and 5 μ g/ml CD28.2 [BD Bioscience, Heidelberg, Germany]) were cross-linked via goat anti-mouse antibodies (7.2 μ g/ml; Dianova, Hamburg, Germany) on 96-well plates (MaxiSorp; Thermo Fisher, Germany). IgG1 and IgG2a antibodies were used as isotype controls (BD Bioscience, Heidelberg, Germany). The anti-phosphocofilin was from Cell Signaling (Leiden, Netherlands) and cofilin antibodies were produced in our own laboratory. PP1 α and NF- κ B antibodies were obtained from Santa Cruz (Heidelberg, Germany).

Imaging flow cytometry. Imaging flow cytometry analysis of the nuclear translocation of NF- κ B was determined as follows. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and fixed by treatment with 1.5% paraformaldehyde for 10 min at room temperature. Subsequently, cells were washed with PBS supplemented with 5% fetal calf serum (FCS), permeabilized with Triton X-100 (0.1% in PBS and 5% FCS, and subjected to nuclear staining (DAPI), pp65 staining (Ser529, clone K10-895.12.50, Alexa Fluor 488 labeled; BD Bioscience), and total p65 staining (anti-p65-phycoerythrin [PE] antibody, Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 30 min at room temperature. Note that the pp65 antibody is suitable for flow cytometry (32). Finally, cells were washed and subjected to imaging flow cytometry analysis as described before (31, 46). Briefly, the content of nuclear p65 was

quantified as the ratio of the p65 fluorescence in the nucleus to the total p65 fluorescence (nuclear p65 score). The nucleus was defined by creating a tight region of interest according to the DAPI staining using the morphology mask of the IDEAS 6.0 software package (Amnis Corp., Seattle, WA). The fluorescence was assessed as mean pixel intensity. Cells with a score of >1.15 were gated as nuclear p65-positive cells. The percentage of positive cells for each sample was determined.

The quantification of T-cell/APC contacts and immune synapse formation was performed as described before (40). Briefly, up to 25,000 cells were acquired with an ImageStream (Amnis, Seattle, WA). Cells were gated for T-cell/APC contacts according to the CD3 and nuclear staining combined with the aspect ratio. A mask that defined the region between the cells combined with the T-cell staining defined the area of the immune synapse. Within this immune synapse, F-actin and LFA-1 staining was quantified as fluorescence intensities.

Superresolution microscopy. Superresolution microscopy (3D-SIM) was performed with an N-SIM Ti-microscope (Nikon, Düsseldorf, Germany) equipped with a 100 \times objective (numerical aperture [NA], 1.49), an Andor camera (DU-987 X-9011), and the NIS-Elements V4.3 software package. Image capturing was adjusted to obtain gray-value signals between 5,000 and 9,000 for each channel (readout speed, 1 MHz).

Sample preparation was as follows. Coverslips (10-mm diameter, 1.5H high precision; Marienfeld, Laud Königshofen, Germany) were placed in 48-well plates and incubated with 0.01% poly-D-lysine (Sigma-Aldrich) at 37°C for 30 min. Meanwhile, Raji cells were incubated with 5 μ g/ml SEB (to stimulate primary T cells) or SEE (to stimulate Jurkat cells) for 15 min. After washing with PBS, 1×10^5 Raji cells were transferred onto the coverslips and incubated at 37°C for 15 min. The coverslips were then washed and saturated using RPMI 1640 containing 10% FCS. T cells (3×10^5) were added, and the cells were centrifuged ($300 \times g$, 2 min). After 45 min, cells were fixed with 1.5% paraformaldehyde and stained with an antibody mixture in fluorescence-activated cell sorting (FACS) wash saponin (FWS) buffer (PBS, 0.5% bovine serum albumin [BSA], 0.1% saponin) as indicated. After three washes in FWS buffer, one in PBS, and one in deionized water, samples were embedded in ProLong Diamond (Life Technologies, Darmstadt, Germany). Image analysis and PCC calculation were performed with NIS-Elements 4.30.02, and superimposition of PCC was executed with Photoshop (PS5).

Quantification of cytokine production. To analyze cytokine secretion, T cells (2 million in 200 μ l) were either stimulated for 24 h or unstimulated. Cells were spun down, and the supernatant was collected. The cytokines and chemokines within the supernatant were analyzed using Bio-Plex assays (Bio-Rad, Hercules, CA). The measurements were carried out according to the manufacturer's instructions using 50 μ l of supernatant. Concentrations were calculated with Bio-Plex Manager 4.1.1.

The amount of intracellular cytokines was analyzed essentially as previously described (22). Briefly, cells were costimulated for 3 days and incubated with brefeldin A for 4 h. Cells were fixed with 1.5% paraformaldehyde, and the intracellular cytokine content was determined using a flow cytometer (LSRII; BD Bioscience).

Statistics. Student *t* tests were performed using GraphPad Prism version 6.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/MCB.00041-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 4, PDF file, 1.0 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.

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We declare no competing financial interests.

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