Regulatory T (Treg) cells require T-cell receptor (TCR) signalling to exert their immunosuppressive activity, but the precise organization of the TCR signalling network compared to conventional T (Tconv) cells remains elusive. By using accurate mass spectrometry and multi-epitope ligand cartography (MELC) we characterized TCR signalling and recruitment of TCR signalling components to the immunological synapse (IS) in Treg cells and Tconv cells. With the exception of Themis which we detected in lower amounts in Treg cells, other major TCR signalling components were found equally abundant, however, their phosphorylation-status notably discriminates Treg cells from Tconv cells. Overall, this study identified 121 Treg cell-specific phosphorylations. Short-term triggering of T cell subsets via CD3 and CD28 widely harmonized these variations with the exception of eleven TCR signalling components that mainly regulate cytoskeleton dynamics and molecular transport. Accordingly, conjugation with B cells indeed caused variant cellular morphology and revealed a Treg cell-specific recruitment of TCR signalling components such as PKC\(\theta\), PLC\(\gamma\)1 and ZAP70 as well as B cell-derived CD86 into the IS. Together, results from this study support the existence of a Treg cell-specific IS and suggest Treg cell-specific cytoskeleton dynamics as a novel determinant for the unique functional properties of Treg cells.

Keywords: Immunological synapse • MELC • Phosphoproteome • TCR signalling • Treg
in Treg cells, thereby, seems to be differentially organized as in Tconv cells: Treg cells show reduced Ca\(^{2+}\) flux and ERK1/2 phosphorylation upon TCR stimulation [6–8], and downstream signalling molecules such as Lck, LAT, and PLC\(\gamma\)1 are essential for Treg cell suppressive capacity [9, 10]. In this line, a novel TCR-mediated ADAP/integrin-independent PLC\(\gamma\)1 activation pathway was described to be required for suppression of Tconv cells by Treg cells [11]. Furthermore, Treg cells exhibit reduced S473 phosphorylation of Akt which seems a prerequisite for suppression [12]. Although the enzymatic activity of the tyrosine kinase ZAP70 seems to be dispensable for the suppressive phenotype of Foxp3\(^+\) Treg cells [13], a single mutation within the SH2-domain of ZAP70 leads to impaired suppressive functions, which indicated its importance at the immunological synapse (IS) [14]. Finally, it has only recently been recognized that the recruitment of TCR signalling components into the IS is differentially organized in Treg cells and Tconv cells. The protein kinase PKK\(\theta\) is recruited to the IS in Tconv cells, in Treg cells, however, this kinase was found to be sequestered away from the IS, but still of importance to control suppression [15].

The spatial recruitment of signalling components during IS formation critically depends on cytoskeleton dynamics, which in turn is controlled by TCR activation and subsequent phosphorylation of proteins regulating cytoskeleton reorganization [5]. As part of these processes the microtubule-organizing centre (MTOC) is rapidly translocated in proximity to the IS. MTOC repositioning depends on LAT, ZAP70 and SLP76 [16] and is regulated by a cascade of distinct isoforms of the family of novel protein kinase C (nPKC) [17]. The MTOC serves as a platform to coordinate molecular movements from and to the IS through the support of microtubule (MT) motors [18]. For instance, TCR microclusters move along MTs towards the centre of the IS in a dynein-dependent manner [19], and hindrance of MTOC polarization, molecular transport and cytoskeleton dynamics prevent proper propagation of TCR signals [20].

It is now tempting to speculate that the localization of signalling modules within the IS may be instrumental for the formation of a Treg cell-specific IS and the suppressive phenotype of Treg cells. At this moment, however, the knowledge on how TCR signalling, protein recruitment and IS formation are differentially organized in Treg cells is far from complete. In the present study, we have now systematically studied the activity and spatial organization of TCR signalling components in ex vivo isolated Treg cells and Tconv cells by employing accurate mass spectrometry and microscopy supported by multi-epitope ligand cartography (MELC) [21, 22]. Together, our data reveal that TCR engagement harmonizes the activity status within the TCR signalling network, but in parallel induces a distinct diverging signalling pattern at regulators of cytoskeletal dynamics. This was accompanied by a variant recruitment of TCR signalling components into the IS upon T cell/B cell conjugation as well as variant CD86 uptake. Thus, results of this study substantiate the concept of a Treg cell-specific IS and suggest subset-specific cytoskeleton dynamics as a novel determinant for their suppressive phenotype.

### Results

**Major TCR signalling components are equally abundant in Treg cells and Tconv cells**

Although various TCR signalling components have already been identified that control differentiation and function of Treg cells [23], a comprehensive picture of the TCR signalling network organization is still missing. Our first goal was to analyse the overall protein abundances of TCR signalling components comparatively in ex vivo isolated, highly pure murine CD4\(^+\)CD25\(^+\) Treg cells and CD4\(^+\)CD25\(^-\) Tconv cells (Supporting Information Fig. 1A). These T cell subsets were spot-checked for major characteristics, i.e. expression of the transcription factor Foxp3 in Treg cells and IL-2 production in Tconv cells (Supporting Information Fig. 2). Next, total proteomes were extracted from sorted Treg cells and Tconv cells and quantitative peptide sequencing was performed using ex vivo iTRAQ labelling and accurate Fourier transform mass spectrometry (FTMS). Database searches were then restricted to high quality MS data and identified >4000 proteins in murine Treg cells and Tconv cells (Supporting Information Table 1). Regulatory data of CD3\(\γ\), PLC\(\gamma\)1, Foxp3, CD4 and CD25 (IL2Ra) were found in full accordance with literature, i.e. equal levels of CD3\(\γ\) and PLC\(\gamma\)1 in both subsets and slightly reduced levels of CD4 and high abundances of Foxp3 and CD25 (IL2Ra) in Treg cells (Fig. 1A, Supporting Information Fig. 3 and Supporting Information Table 1). Cross-inspection of our data with former published proteomic studies [24–26] could now substantiate a series of proteins with Treg cell-specific abundances, although these proteins are not described to be directly involved in TCR signalling. This includes Helios, Aiolos, Sortin nixin-18, Ergic1, Annexin A4, CAPG, Stim2, NDRG1, Ladinin-1, Niban, S100A4 and Reticulocalbin-1 (Supporting Information Table 1).

With respect to TCR signalling we mapped our proteome data with the support of interactome databases (GeneGo, MetaCore and IPA) and confirmed that the canonical TCR signalling pathway was completely covered. Notably, all TCR signalling components were found with similar abundances in both T cell subsets with the exception of Themis. In Treg cells, we detected Themis with reduced protein levels (Fig. 1A, Supporting Information Fig. 3 and Supporting Information Table 1), a finding which was confirmed by Western blotting and at the mRNA level by quantitative RT-PCR (Supporting Information Fig. 4A). Themis acts downstream of LAT and is required for IL-2 responses in Tconv cells [27]. Hence, we speculated that reduced Themis expression in Treg cells might be causal for known anergic signalling and impaired IL-2 responses. However, retroviral overexpression of Themis in ex vivo isolated Treg cells did neither restore IL-2 production nor abrogate suppressive capacity of Treg cells (Supporting Information Fig. 4B and 4C), suggesting that reduced Themis expression is not critical for the functional properties of Treg cells. In summary, our MS analysis revealed that major TCR signalling components are equally abundant in both T-cell subsets.
Identification of diverging TCR signalling components

We next asked, whether equally abundant TCR signalling components might have a differential activity in Treg cells and Tconv cells. Protein phosphorylations constitute the key mechanism in signalling and we therefore aimed to profile the TCR signalling network by using quantitative phosphoproteomics. Our pivotal aim was to study the responsiveness of the TCR signalling components under rapid and robust activation conditions. For that, Treg cells and Tconv cells were ex vivo isolated and batch-wise either left untreated or stimulated for 5 min using anti-CD3 and anti-CD28 antibodies (Supporting Information Fig. 1B). Phosphorylated peptides of these four biological samples were then isolated and comparatively analysed by quantitative peptide sequencing using iTRAQ labelling and accurate FTMS. Data evaluation only considered high-confident phosphopeptides that iTRAQ-FTMS unambiguously detected in all four biological samples. This approach identified 3756 phosphorylations in freshly isolated Tconv cells and Treg cells (Supporting Information Table 2).

About one-third of these phosphorylation sites could be assigned to major signalling pathways covering the TCR signalling network. To the best of our knowledge, we found all phosphorylations of the canonical TCR signalling pathway perfectly in line with the literature. For instance, phosphorylations at immunoreceptor tyrosine-based activation motifs (ITAMs) of Tconv cells (CD3ε, CD3ζ) and CD3/CD28-responsive down-stream components (ZAP70, SLP76) in Treg cells (Fig. 1B and Supporting Information Fig. 3B). Interestingly, the profiles of the corresponding tyrosine phosphorylations at CD3ε and CD3ζ in Treg cells revealed first differences between both T cell subsets. They were found not inducible and with similar high intensities in non-stimulated and stimulated Treg cells.
Figure 2. Phosphoproteomic profiling identifies converging and diverging TCR responses. For quantitative phosphoproteomics, ex vivo isolated Treg cells and Tconv cells were CD3/CD28-stimulated for 5 min or left non-stimulated and analysed by using quantitative MS. All regulation factors are given in log2 scale. (A) Box plots show regulations of all phosphorylation sites of proteins from stimulated Treg cells or Tconv cells in relation to non-stimulated cells. Numbers indicate the significantly up- or down-regulated phosphorylation sites when assuming a normal distribution. (B) Comparison of up- and down-regulated phosphorylation sites in Treg cells and Tconv cells (from A). (C) Scatter plot depicting regulations of all phosphorylated peptides in Treg cells (x-axis) and Tconv cells (y-axis). One hundred and twenty one phosphopeptides that are significantly differentially regulated between Treg cells and Tconv cells (see Supporting Information Fig. 5) are indicated by red dots. (D) Heat
(Fig. 1B and Supporting Information Fig. 3B). This indicates a pre-activated phenotype of the TCR signalling network in freshly isolated Treg cells, which likely is a consequence of tonic interactions with antigen-presenting cells in vivo [28]. Using Western blotting analysis we could indeed confirm the enhanced level of phosphorylated CD3ζ in non-stimulated Treg cells in comparison to non-stimulated Tconv cells (Supporting Information Fig. 5). Nevertheless, this pre-activation did not impair the responsiveness of the TCR signalling network in Treg cells as illustrated by the induced Y492 phosphorylation of ZAP70 (Fig. 1B and Supporting Information Fig. 3B). In a global view, TCR triggering in Treg cells causes similar numbers of induced and repressed phosphorylation events, whereas Tconv cells showed a higher number of significantly up-regulated phosphorylations (Fig. 2A). Several TCR components exhibited similar phosphorylation responses in both T cell subsets, although the number of jointly up- and down-regulated proteins in Treg cells and Tconv cells is limited (Fig. 2B). This includes the aforementioned ZAP70 as well as known TCR scaffold proteins SASH3/Sly, raflin, Lad/TSAd and LIME1, indicating an overall similar architecture of the signal networks. The vast majority of CD3/CD28-responding phosphorylations exhibited subset-specific regulations and a comparative inspection revealed a total of 121 phosphorylations (Supporting Information Fig. 6) that discriminate Treg cells and Tconv cells in the first five min of signalling (Fig. 2C and Supporting Information Table 2, sheet3). From a mechanistical angle these Treg cell-specific regulations can be grouped in six classes (Fig. 2C and D). For instance, the deubiquitinase CYLD, which plays a role in the IS recruitment of Lck, was induced at its inhibitory S414 site only in Tconv cells (class I). Likewise, class II phosphorylations are predominantly induced only in Treg cells; comprising the activation site T538 of PKCθ (Fig. 1C) as well as components of gene regulation (ISW1 and ROA1) and vesicle trafficking (AP381 and α-PIX).

In a side-by-side inspection of both subsets, the majority of 121 phosphorylations (>60%), showed an opposite regulatory behaviour under the same stimulation condition (Fig. 2D, classes V and VI). However, when non-stimulated Tconv cells served as the reference for normalization, protein-specific phosphorylations became apparently similar after five min stimulation (Fig. 2E, compare lanes five min). This convergence of phosphorylations is most apparent but not restricted to class V and VI proteins. For instance, the abundance of phosphorylated T538 of PKCθ (class II) was at its lowest in freshly isolated Treg cells, but converged to similar activation level after TCR engagement in both stimulated subsets. Thus, equal ex vivo TCR stimulation of Treg cells and Tconv cells notably harmonize the post-translational status of the TCR signalling network.

Nonetheless, a distinct set of eleven TCR signalling components were not harmonized by TCR engagement and indicated an even diverging signalling pattern in Treg cells and Tconv cells (Fig. 2F and MS data for DBNL, MAPK14 and TIAM in Fig. 1C). This group is composed of known TCR signalling components and, from a systematical view, is enriched for one functional class (Table 1): Beside the hematopoietic lineage-specific phosphatase PTPN18 and the Transducin-like enhancer of split 3 (TLE3), both involved in T cell differentiation [29–32], all other nine components are associated with cytoskeleton dynamics [19, 33–39]. Many of those proteins were found with induced phosphorylations only in Tconv cells, whereas these responses are even repressed in Treg cells. This includes DC1L1/LIC1, a subunit of the dynein complex, that is supposed to determine cargo load [40]. In contrast, Treg cell-specific induced phosphorylations were identified at SMCR8 and TIAM, of which the latter is required for LFA-1 integrin activation [41].

In conclusion, ex vivo Treg cells exhibited a pre-activation TCR phenotype. Their phosphorylation status discriminates them from Tconv cells, but can be largely harmonized under precise TCR activation conditions ex vivo. For that, phosphorylations showed opposite regulations in Treg cells and Tconv cells and the global phosphorylation signatures widely converge in only 5 min. Still, a set of eleven components that are mostly reported as regulators of the cytoskeleton identified diverging nodes in the two subset-specific TCR signalling networks. In this context, protein network analyses based on induced phosphorylated proteins actually confirmed the close interconnectivity between TCR signalling and the cytoskeleton (Supporting Information Fig. 7A). Thereby, the responding Tconv cell network revealed the superior enrichment of “TCR signalling components”, whereas the responding Treg cell network revealed the superior enrichment of “cytoskeleton dynamics”. Indeed, network analyses restricted to the 121 subset-specific phosphorylations strengthened the hypothesis that mainly TCR signalling and cytoskeleton dynamics discriminate Treg cells from Tconv cells (Supporting Information Fig. 7B).

**Treg cells reveal unique cell polarization and MTOC positioning but do not recruit PKCθ to the IS**

Diverging signalling components indicated a variant regulation of cytoskeletal organization in the two T cell subsets that may orchestrate T cell motility, conjugation, IS formation as well as...
confirmed the MTOC positioning phenotype. In Treg cells, this approach (class II). Thus, we next investigated PKC θ was identified as a Treg cell-specific effector functions. Here, we focused on conjugation and IS formation. For that, Treg cells and Tconv cells were isolated from ovalbumin (OVA)-TCR transgenic mice (DO11.10), IS formation was induced by OVA peptide-loaded ex vivo isolated B cells, and polarization dynamics of BT-pairs were studied by microscopy. Global inspection of BT-pairs already demonstrated a subset-specific morphology phenotype. Along cell conjugation the shape of Tconv cells was found to be more elongated as those from Treg cells, which exhibited a more rounded morphology upon conjugation (Fig. 3A). We next considered only those BT-pairs, which had established a mature actin-enriched IS as indicated by phalloidin. Cells were additionally stained for the microtubule network and we found the distance between MTOC and IS significantly diminished in conjugated Treg cells as compared to Tconv cells. To score differences within the repositioning of the MT network and especially the MTOC in Treg cells and Tconv cells, we determined the polarization index [42] upon BT-pairing and confirmed a significant Treg cell-specific polarization phenotype and MTOC positioning (Fig. 3B).

MTOC positioning is regulated by PKC family members [16], and PKCθ was already reported to delocalise from the Treg cell-IS [15]. Interestingly, PKCθ was identified as a Treg cell-specific component following TCR engagement in our phosphoproteomic approach (class II). Thus, we next investigated PKCθ localization dynamics in relation to MTOC positioning (Supporting Information Fig. 1C). IS formation was studied by using anti-CD3/anti-CD28-coated coverslips, and confocal spinning disk microscopy confirmed the MTOC positioning phenotype. In Treg cells, this MTOC positioning phenotype was not accompanied with a notable recruitment of PKCθ to the IS in the first 30 min (Fig. 3C and D). Thus, PKCθ is not actively delocalized away from the Treg cell-IS but instead is not recruited at all. In contrast, for Tconv cells we could confirm IS recruitment of PKCθ that became more obvious after 3D reconstruction (Fig. 3C and D). Interestingly, PKCθ recruitment into the Tconv cell-IS was only found transiently after ten min, and already 30 min after TCR activation the majority of cells lost the polarized PKCθ distribution (Fig. 3C). Thus, MTOC positioning in Tconv cells is accompanied by a transient IS recruitment of PKCθ, whereas an even more polarized positioning of the MTOC towards the IS in Treg cells could not attract PKCθ into the synaptic compartment.

Treg cells establish a unique transport phenotype of TCR signalling network components at the IS

Since we (i) revealed Treg cell-specific and diverging signalling of cytoskeleton regulators and (ii) characterized a variant MTOC positioning and impaired PKCθ recruitment in Treg cells, we next aimed to study the spatial organization of selected TCR signalling components in stimulated Treg cells and Tconv cells. For this, we employed the multi-epitope ligand cartography (MELC) technology [21, 22], and evaluated a total of about 300 OVA-specific BT-pairs using a panel of 25 antibodies (see Material and Methods, Supporting Information Fig. 1C and Supporting Information Video 1). This approach allowed subcellular localization analyses

Table 1. Intrinsic Treg cell-specific phosphoresponses

| Uniprot | Trivial names | Site  | Treg cells | Tconv cells | Function | Node |
|---------|---------------|-------|------------|-------------|----------|------|
| STMN1   | Stathmin      | S16   | −          | ++++        | Cytoskeletal reorganization | tubulin / MTOC [33] |
| TLE3    | TLE3          | S217  | −          | ++++        | Transcriptional co-repression | CaMKII [53] |
| DC1L1   | LIC1          | S412  | −          | ++++        | Vesicle transport | TCF / Lef [31] |
| RHG15   | ArhGAP15      | S51   | −          | ++++        | TCR microcluster dynamics | FoxA1 / HDAC1/2 [32] |
| PTN18   | FPTN18        | Y381  | −−         | +++        | Cytoskeletal reorganization | Dynemin [19] |
| M3K3    | MAP3K3        | S337  | −          | +++        | T cell differentiation / activation | tubulin/MTOC [34] |
| DBNL    | Drebrin-like  | S277  | −−         | +++        | Cytoskeletal reorganization | small GTPases (Rac) [78] |
| MK14    | MAPK14        | Y182  | −−−        | ++         | induced Treg cell generation | Pak1/2 [54] |
| ML12B   | MLC20         | S20   | −−         | ++         | Cytoskeletal reorganization | Lck / ZAP70 [29] |
| SMCR8   | SMC8          | S416  | ++         | −          | vesicle transport | IFN-γ [79] / NFκB [80] |
| TIAM1   | TIAM1         | S1437 | ++         | −          | integrin signalling | PLCγ [55] |

List of 11 TCR signalling components that were not harmonized upon CD3/CD28-stimulation and that revealed an even diverging signalling pattern in Treg cells and Tconv cells. Regulations of phosphorylation sites in Treg cells and Tconv cells are given by “+” and “−” signs (−/+: 0.00-0.50; −−/+:+: 0.50-1.00; −−−/: 1.00-1.50; −−−−/+++: >1.50 (log2 scale)), known functional roles in T cells and major nodes are listed.
regions. Our segmentation strategy, which was based on the position of key TCR signalling components as well as their specific phosphovariants. Following 30 and 120 min after BT-pair formation, we could define the SMAC and cSMAC as well as the entire IS that spanned the width of the SMAC and protruded five pixels into both the B and T cell (Fig. 4A and B, and Supporting Information Fig. 8). Stable BT-pairs were defined based on signals from CD3ε, CD45R, F-actin, pSLP76 and pLAT and allowed recording the position and size of a mature IS (Fig. 4A).

At first glance, the basic IS architecture of BTreg- and BTconv-pairs appeared similar as indicated by the global intensity pattern of the inspected TCR signalling components (Fig. 4C, compare Treg cells versus Tconv cells at 30 and 120 min). The MELC analyses also confirmed the accumulation of activated proximal TCR signalling components, i.e. pCD3ε, pZAP70, pLck, pLAT, pSLP76 and pPLCγ1 at the IS, SMAC and cSMAC. This accumulation was found to further increase after 120 min and coincided with the enrichment of the corresponding total protein levels within these regions as well (Fig. 4C). Statistical analyses of the BT-pairs revealed a number of striking differences in IS organization: (i) Treg cells were notably impaired in recruiting additional CD3ε, Lck, SLP76 and PLCγ1 after the formation of a mature IS at 30 min. In contrast, Tconv cells were able to continue this recruitment over a period of 30 to 120 mins more efficiently (Fig. 4D, relative differences over time). Interestingly, both subsets likely continue signalling along this period as observed by the consecutive accumulation of the corresponding phosphovariants. (ii) Significantly lower amounts of PLCγ1, CD3ε and pZAP70 were detected at the Treg cell-IS (Fig. 4E, relative differences in subsets). Thereby, pZAP70 was mostly affected after 30 min but remained reduced in the cSMAC, and PLCγ1 and CD3ε were found significantly reduced only after 120 min (Fig. 4E, and representative images in Fig. 4F and G). (iii) Instead, significantly higher amounts of CD45, Lck, SLP76 and Lyn as well as pERK, pPLCγ1 and pLAT were localized at the Treg cell-IS 30 min after conjugation (Fig. 4E). This argues for a more rapid maturation of the Treg cell-IS compared to the Tconv cell-IS. (iv) Only Treg cells were able to recruit CD86 and CD80 to their IS compartment (Fig. 4E and Supporting Information Fig. 6), as described previously [43]. Thus, CD86 transendocytosis, as a part of the Treg cell suppressive phenotype, was now demonstrated 30 min after IS formation and did not indicate any exhaustion after 120 min.

In conclusion, MELC analyses showed different recruitment of TCR signalling components into the IS of Treg cells versus Tconv cells at early time points, which, however, ultimately resulted in a more alike distribution at 120 min. Still, the key signalling components CD3ε, ZAP70, PLCγ1 and CD86 remained differentially distributed at the IS and could explain Treg cell- and Tconv cell-specific phenotypic responses.

Discussion

TCR signalling controls differentiation and function of Treg cells [23]. Although previous studies already had described some differences in TCR signalling between Treg cells and Tconv cells [44], a comprehensive picture of the TCR signalling network of pSLP76 in the actin-rich IS (green dot, Fig. 4A and B), could define the SMAC and cSMAC as well as the entire IS that spanned the width of the SMAC and protruded five pixels into both the B and T cell (Fig. 4A and B, and Supporting Information Fig. 8). Stable BT-pairs were defined based on signals from CD3ε, CD45R, F-actin, pSLP76 and pLAT and allowed recording the position and size of a mature IS (Fig. 4A).
Figure 4. Activation-dependent recruitment of TCR signalling components to the is. BT-pairs were generated as in Fig. 3, and pairs were fixed after 30 or 120 min and analysed by sequential staining (multi-epitope ligand cartography, MELC). (A) Representative image showing a BT-pair with pSLP76 (green), CD3ε (red) and F-actin (gray) staining that was used to define the different compartments of the IS (IS circled by dotted line, SMAC framed by dashed line and cSMAC framed by solid line). (B) Segmentation strategy of the IS compartments as determined by the position of pSLP76 staining (green dot), which was used to define a three pixel (0.86 μm) wide SMAC region stretched over the synapse as defined by F-actin. The cSMAC was defined as the middle 33% of the width of the SMAC. The entire IS area was defined as an oval spanning the width of the SMAC and protruding five pixel (1.43 μm) beyond the SMAC into either cell. (C) Relative intensities of all analysed (phospho)proteins in BTreg- and BTconv-pairs 30 and 120 min after pair formation determined in the B cell (B) and T cell (T) without the IS, the B cell (Bs) and T cell (Ts) part of the IS without SMAC, the entire IS, the complete SMAC (S) and the cSMAC (cS). Note the gradual enrichment of both total TCR-signalling proteins and their phosphorylated variants in the IS (IS, S and cS). (D,E) Relative differences of all analysed (phospho)proteins in BT-pairs in the IS (i.e. IS, S and cS). Only statistically significant differences are shown (p < 0.05, two-tailed t-test). The relative differences and p-values for all BT-pair compartments are given in Supporting Information Fig. 8. (F,G) Representative images showing the localization of proteins used to define the IS compartments of BTreg- (F) and BTconv- (G) pairs (upper images; CD3ε (red) and pSLP76 (green), Foxp3 (blue) marks only Treg cells) and representative images showing differences between BTreg- and BTconv-pairs regarding reduced PLCγ1 (green) and pZAP70 (red) localization within the IS and Ts, and the cSMAC, respectively (lower images). F-actin is shown in gray. A photomontage of BT-pairs (BTreg vs. BTconv) that includes information of all investigated proteins and phosphoproteins obtained by MELC is given in Supporting Information Video 1. Representative images given in A,F,G are from one of two independent experiments with eight mice each and of 51 BTreg- versus 130 BTconv-pairs at 30 min and 28 BTreg- versus 62 BTconv-pairs at 120 min analysed, heat maps shown in C-E represent pooled data from all analysed BT-pairs.

organization was missing. Results of this study now suggest a Treg cell-specific activation of cytoskeleton regulators and a subsequent Treg cell-specific recruitment of TCR signalling components to the mature IS. This constitutes a mechanistic model of how functionally distinct TCR signalling is realized in both T cell subsets.

First, the proteome analyses determined largely identical abundances of TCR signalling components within Treg cells and Tconv cells. This observation is widely in line with recent data from proteome studies of human and murine Treg cells, although those studies did not specifically focus on TCR signalling [24–26]. The present proteomic approach identified low abundance of Themis in Treg cells, a molecule known to play a role in the early LAT signalling in Tconv cells [27]. Overexpression of Themis, however, could neither restore IL-2 expression nor reduce the suppressive
capacity of Treg cells in vitro. Nevertheless, Themis levels play a role in T cell development and survival [45–48], and Themis has been reported to modulate IL-10 expression [49], aspects that were not addressed in this study. Thus, protein abundances seemed not to be the key determinant of functionally distinct TCR signalling networks.

In contrast, this first phosphoproteome study on primary murine T cells detected numerous Treg cell-specific phosphorylations in the TCR signalling network. Ex vivo Treg cells displayed a pre-activated phosphorylation status of key TCR signalling molecules (e.g. CD3ε, CD3ζ and SLP76). Initially, this appeared counterintuitive since a diminished tyrosine phosphorylation of CD3ζ in peripheral human Treg cells has been reported [7, 8]. However, T cells residing in secondary lymphoid organs - as used in our study - continuously receive “tonic” receptor stimuli [50] that can result in priming the down-stream signal network as described for Tconv cells [28], and this now could be confirmed here for Treg cells as well. The functional role of this priming needs further investigation, but it did not abrogate Treg cells’ responsiveness since we found Y492 in ZAP70 equally well phosphorylated in ex vivo isolated T cell subsets and equally well inducible upon TCR engagement. This site is a known substrate site of Lck and serves as an autophosphorylation site of activated ZAP70 as well [51]. The CD3ζ immunoreceptor tyrosine-based activation motif (ITAM), however, was already found phosphorylated in ex vivo isolated, non-stimulated Treg cells. Hence it is fair to speculate that the signal amplification mechanism involving the dissociation of ZAP70 from the TCR complex, as very recently described for Tconv cells [52], is already induced in non-stimulated Treg cells. This would support ZAP70 function in inside-out signalling and adhesion of Treg cells, which did not require its kinase activity [13].

In total, 121 Treg cell-specific phosphorylations define known and novel down-stream components of the TCR signalling network. Identical stimulation conditions thereby converge the phosphopatterns significantly within five min. More noteworthy, harmonizing of both TCR signalling networks is predominantly realized by counter-regulated signalling. This involved well-known proximal TCR signalling components like PKCθ, SLP76 and GRP2/CaI DAG-GEFI, and substantiates the model of Treg cells' responsiveness since we found Y492 in ZAP70 equally well phosphorylated in ex vivo isolated T cell subsets and equally well inducible upon TCR engagement. This site is a known substrate site of Lck and serves as an autophosphorylation site of activated ZAP70 as well [51]. The CD3ζ immunoreceptor tyrosine-based activation motif (ITAM), however, was already found phosphorylated in ex vivo isolated, non-stimulated Treg cells. Hence it is fair to speculate that the signal amplification mechanism involving the dissociation of ZAP70 from the TCR complex, as very recently described for Tconv cells [52], is already induced in non-stimulated Treg cells. This would support ZAP70 function in inside-out signalling and adhesion of Treg cells, which did not require its kinase activity [13].

In total, 121 Treg cell-specific phosphorylations define known and novel down-stream components of the TCR signalling network. Identical stimulation conditions thereby converge the phosphopatterns significantly within five min. More noteworthy, harmonizing of both TCR signalling networks is predominantly realized by counter-regulated signalling. This involved well-known proximal TCR signalling components like PKCθ, SLP76 and GRP2/CaI DAG-GEFI, and substantiates the model of Treg cells having a variant activation status in vivo. Furthermore, this demonstrates that Treg cells in vivo integrate signals from multiple receptors and have the capacity to reverse the direction of TCR-mediated regulations. It was exciting to detect even a diverging TCR signalling under the applied stimulation conditions in a set of eleven proteins. These components have been related previously to distal parts of the TCR signalling network. Nine of these are regulators of cytoskeleton dynamics and molecular transport [19, 33, 37, 53–55] and indeed cellular polarity, MTOC positioning and signal component recruitments then confirmed a cell-polarization as well as an IS assembly phenotype of Treg cells.

Such an IS phenotype is still mostly defined by PKCθ that plays a pivotal role in MTOC dynamics and IS formation in T cells [56]. Zanin-Zhorov and colleagues recently observed PKCθ to be delocalized six min after Treg cell-IS formation and gave the first evidence for a Treg cell-specific IS organization [15]. Here, we could now confirm this phenotype and further demonstrate that in contrast to Tconv cells, PKCθ is not recruited at all to the Treg cell-IS, whereas other TCR components were found efficiently recruited. Actually, in Tconv cells PKCθ is recruited only transiently to the IS by so far unknown antagonistic translocation mechanisms. This finding must be further elucidated e.g. in bi-cellular conjugations, but likely is under control of post-translational modifications. During TCR-induced signalling, PKCθ integrates co-stimulatory signals from CD3 and CD28, and GLK (germinal centre kinase-related protein kinase) can phosphorylate PKCθ at T538, which coordinates its activity and localization [57, 58]. Interestingly, we found this site solely inducible in Treg cells. Furthermore, PKCθ localization might depend on sumoylations [59], which was not addressed in the present study. But what is the role of delocalized PKCθ in the context of Treg cell-IS functions? In Tconv cells, upon TCR stimulation PKCθ supports NF-kB activation [60] and can maintain phosphorylated RelA, the p65 subunit of NF-kB, in the nucleus [61]. Interestingly, Treg cells were found to accumulate phosphorylated p65 at the late IS, although its functional role there remains elusive. In the same pathway, PKCθ is described as an interacting antagonist of the deubiquitase CYLD, which acts on Lck to support its IS localization [62, 63]. We found CYLD only responding in Tconv cells, indicating that the PKC/CYLD axis is differentially organized in Treg cells.

A more systematic inspection of the Treg cell-IS was corroborated by MELC analyses. When compared to Tconv cells, about one-third of the here-inspected components displayed significantly different recruitment dynamics to the mature IS. Phosphovariants of TCR signalling components were equally recruited into the IS of Treg cells and Tconv cells as expected, however, Tconv cells could recruit TCR signalling components more efficiently. It is tempting to speculate that a limited recruitment of these components can dampen TCR signalling in Treg cells. Indeed, ZAP70 phosphorylated at Y318, a site unambiguously needed in T cell activation [64], was reduced in the IS in BTreg-pairs at 30 min, and this holds true for the cSMAC even after 120 min. Furthermore, we observed reduced levels of PLCγ1 in the Treg cell-IS 120 min after BT-pair initiation. Mechanistically this could be explained by the differentially regulated S210 in the P1 domain of SLP76 that facilitates the interaction with the SH3 domain of PLCγ1 and is required for TCR-mediated activation [65]. LAT competes for PLCγ1 interaction via this SH3 domain, and this PLCγ1/LAT complex is restricted to the cell membrane in resting Jurkat T cells [66]. Thus interfering with SLP76/PLCγ1 interaction through S210 phosphorylation could result in aberrant complex recruitment via LAT to the Treg cell-IS. Here, one should note that, due to limitations in the antibody panel used in the MELC analyses, we most probably underestimate the number of proteins that show differences in IS recruitment. For instance also Lymphocyte Cytosolic Protein 1 (LCP1 or L-plastin/PLSL) might be differentially recruited into the Treg cell-IS. Phosphorylation at S5 supports its translocation to the IS and therewith its contribution to cell motility in Tconv cells [67]. This site was indeed induced in Tconv cells as expected, however, in Treg cells the S5

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phosphorylation was down-regulated, indicative for differential localization and activity status of LCP1 in Treg cells.

Importantly, we observed an enrichment of CD86 and, to a lesser extent, CD80 at the Treg cell-IS. It became apparent that transendoctyosis of CD86 and CD80 by CTLA-4 in activated T cells acts to inhibit CD28 costimulation by cell-extrinsic depletion of these ligands [43]. In induced Treg cells, the acquisition of CD86 occurs to a higher extent than that of CD80 [68], which is now corroborated by the MELC analysis showing enrichment of both CD86 and CD80 in Treg cells although with different amounts. In addition, a series of combined stimulations using CD80/CD86 B cell clones and CD28/CTLA-4/PL-1L-expressing Treg cell or Tconv cell clones demonstrated a CD86-dependent difference in Treg interaction strength and Treg cell activation [69]. Together with the notion that T cells lacking PKCθ show a hyperstabile IS [56] and the here-described absence of PKCθ in the Treg cell-IS, this points to an CD86/PKCθ axis that organizes the stability of cell conjugates.

But which mechanisms could orchestrate the Treg cell-specific recruitment of these TCR signalling components? Here, this study indicates the involvement of eleven candidates several of which known to regulate cytoskeleton dynamics and molecular transport in T cells: (i) For instance, S16 phosphorylation of stathmin is triggered by CD28 co-stimulation [53] and regulates MT dynamics and MTOC polarization [33]. (ii) TIAM1 influence cytoskeleton dynamics via the activation of GTPases. Interestingly, TIAM1 was reported to control T cell trafficking by regulating TIAM1-PKCθ interaction at the leading edge of the polarized T cell [70]. (iii) MAPK14 can be phosphorylated by ZAP70 in an alternative activation pathway in response to TCR signalling in T cells and is involved in MTOC positioning at the IS as well as in T cell activation [71, 72]. (iv) Drebrin-like/HP-55 knock-out mice showed defective T cell proliferation, cytokine production and TCR activation [36], and can itself decrease TCR localization at the IS [37]. (v) Finally, LIC1 of the dynein complex bridges the motor domain to its cargo [40], and it is now tempting to speculate their roles in TCR microcluster transport along MT towards the centre of the IS [19].

In conclusion, cellular polarization and molecular transport of TCR signalling components to the IS discriminate activated Treg cells from Tconv cells. These findings suggest that selective targeting of molecular transport in Treg cells might be exploited in the future as a novel therapeutic strategy to modulate the immunosuppressive phenotype of this clinically relevant cell type.

**Materials and methods**

**Mice**

BALB/c mice were purchased from Harlan or Janvier, and TCR-transgenic DO11.10 as well as Foxp3<sup>Cre<sub>CD2</sub></sup> reporter mice were bred under specific pathogen-free conditions at the animal facility of the Helmholtz Centre for Infection Research (Braunschweig, Germany). Mice were housed and handled under license number 325.1.53/56.1-HZ1 (City of Braunschweig, Veterinary Department) in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. Harvesting of murine cells/organs was performed in compliance with §4 of the German animal protection law (TierSchG BGBI S. 1308; 28.07.2014). Number of animals used were notified to the Lower Saxony State Office for Consumer Protection and Food Safety according to the German laboratory animal reporting act (VerwvTierMeldV BGBI S. 4145; 12.12.2013).

**Antibodies**

Flow cytometry was performed using the following antibodies from eBioscience: anti-CD4 (RM4-5), anti-CD25 (PC61.5), anti-hCD2 (RPA-2.10), anti-Thy1.1 (HI31), anti-IL-2 (JE56-5H4) and anti-Foxp3 (FJK-16s). MELC was performed using the following antibodies from BD Pharmingen against total or phosphorylated proteins: CD3e (17A2), CD4 (RM4-5), pCD3 (pY142; K25-407.69), Lck (MOL-171), pLck (4/Lck-pY505), pLAT (pY171; I58-1169), PLCγ1 (10/PLCgamma), pPLCγ1 (pY783; 27/PLC), SLP76 (H3), sSLP76 (pY128; J141), pZAP70/STK (pY319/pY352; 17A), pERK1/2 (pT202/pY204; 20A); pS6 (pS235/pS236; N7-548), NF-κB pp65 (pS529; K10.895.12.50), pp38 MAPK (pT180/pY183; 36), CD45 (30-F11), CD45R (RA3-6B2), MHCII I-A/I-E (2G9), CXCR4/CD184 (2B11), Foxp3 (MF23) and actin (C4). Antibodies against CD86 (GL-1), CD80 (16-10A1) and tubulin (TU-01) were obtained from Biologend. An antibody against Fyn (15) was obtained from Santa Cruz, and against ERK1/2 (16-283) from Millipore. Propidium iodide (PI; Sigma) served for nuclear staining. Western blotting was performed using antibodies against Themis (polyclonal, Abcam), β-actin (AC-74, Sigma), phosphotyrosine (4G10, Millipore) and pCD3 (pY142; K25-407.69, BD Biosciences). For standard epifluorescence and confocal spinning disk microscopy antibodies against α-tubulin (clone α3A2, Synaptic Systems) and PKCθ (sc-212, Santa Cruz) were used. Alexa Fluor®-labelled secondary antibodies and phalloidin were purchased from Molecular Probes.

**Isolation of murine CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>−</sup> T cells and TCR stimulation**

Single-cell suspensions of spleen and lymph nodes from BALB/c or DO11.10 mice were enriched for CD4<sup>+</sup> T cells using microbeads and the autoMACS magnetic separation system (Miltenyi Biotec). Enriched CD4<sup>+</sup> T cells were stained with anti-CD4 and anti-CD25 antibodies and subsequently separated into CD4<sup>+</sup>CD25<sup>−</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells by fluorescence-activated cell sorting (FACS) on a FACSaria (BD Biosciences).

For TCR triggering, purified T cells were incubated with Biotin-labelled anti-CD3s (clone 145-2C11; BD Biosciences) and anti-CD28 (clone 37.51; BD Biosciences) antibodies on ice and resuspended in complete RPMI media at 2.5 × 10<sup>6</sup> cells/500 μL. Two
minutes before crosslinking via addition of 50 μg streptavidin, the cell suspension was pre-warmed in a 37°C water bath. T cell activation was stopped after 5 min by adding a twentyfold excess of ice-cold PBS and subsequent handling on ice or 2°C. Cell pellets were stored in a nitrogen gas phase until further use.

Flow cytometry

Cells were stained with LIVE/DEAD Fixable Dead Cell stains (Invitrogen), followed by fluorochrome-conjugated antibody staining. To reduce unspecific antibody binding, surface and intracellular stainings were performed in the presence of anti-CD16/CD32 (2.4G2, BioXCell) antibodies and ChromPure rat IgG whole molecule (Jackson ImmunoResearch), respectively. For IL-2 cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (Sigma) and ionomycin (Sigma) for 4 h, and brefeldin A (Sigma) was added for the last 2 h. Cells were fixed, permeabilised and stained intracellularly for Foxp3 using the Foxp3 staining set (eBioscience). Samples were acquired with a LSR II (BD Biosciences) and analysed using FlowJo software (Tree Star Inc). Doublets and dead cells were excluded from flow cytometry analysis.

Cell lysis and protein digest

Purified cells were resuspended in 50 mM HEPES (pH 7.5) complemented with 250 mM NaCl, 1 mM EGTA, Complete Protease inhibitor (Roche), 10 mM NaF, 2.5 mM Na3VO4, 50 ng/ml calyculin A, 1 % phosphatase inhibitor mixes Sigma 1 and 2, and 1 % Triton X-100. Cell lysis and DNA degradation was supported by sonication (Branson Sonifier) and proteins were isolated as previously described [73]. Total protein amounts were estimated by applying a small aliquot of the sample onto an SDS-gel, staining with colloidal Coomassie, and integration to 500 ng BSA as a standard by using AIDA software (Raytest, version 4.06).

Proteins were digested in 50 mM triethylammonium bicarbonate (TEAB) containing 10 % acetonitrile (ACN) and phosphatase inhibitors. A protein/protease ratio of no more than 50:1 was applied and digestion was performed at 37°C overnight. Peptides were vacuum dried, resolved in 0.2 % trifluoroacetic acid (TFA) in water, desalted on self-packed Lichroprep RP18 (10 μL, Merck), eluted in 0.2 % TFA in 60 % ACN and dried again.

Isolation of phosphopeptides, iTRAQ labelling and SCX chromatography

Phosphopeptides were isolated in two consecutive steps using Phos-Select iron affinity gel (Sigma) (Step 1) and Ga-IMAC (Step 2). Desalted peptides were resolubilised in 1 mL binding buffer containing acetonitrile, methanol, H2O and acetic acid (3:3:3:1). The 200 μL Phos-Select gel was added and peptides were allowed to bind for 3 h on a rotating wheel. Beads were washed eight times with 600 μL binding buffer and fractions were reconcentrated for Ga-IMAC. The Phos-Select gel was then washed four times with 600 μL binding buffer, four times with 600 μL binding buffer containing 200 mM NaCl, two times with H2O and phosphopeptides were eluted five times by using 500 μL 1 M KH2PO4. Eluates were acidified to a final concentration of 0.2 % TFA and desalted.

iTRAQ labelling was performed according to the manufacturer’s protocol (Applied Biosystems). Briefly, 150 μg dried peptides was redissolved in iTRAQ dissolution buffer at a ratio of no more than 50 μg peptide/20 μL buffer and incubated with three vials of label for 2 h followed by vacuum drying. A small aliquot was used to confirm the minimum percentage of peptide labelling using a 30 min RP18 gradient and the OrbitrapVelos mass spectrometer. Labelled fractions were combined and subjected to SCX chromatography to generate phosphopeptide fractions for 15 independent LC-MS analyses. The following iTRAQ reporter combinations were used: mouse proteome (Treg cells 115, Tconv cells 117) and mouse phosphoproteome (Treg cells resting 114, Treg cells stimulated 116, Tconv cells resting 115, Tconv cells stimulated 117).

The complex peptide mixtures were than subfractionated according to Reinh et al. [74]. In short, up to 100 μg peptide were separated on a Mono S PC 1.6/5 column (GE Healthcare) via an Etan micro-LC system (GE Healthcare) using a 30 min gradient from 0 to 35 % SCX buffer B (0.065 % formic acid (FA), 25 % ACN supplemented with 0.5 M KCl) at a flow rate of 150 μL/min. Up to twenty 150 μL fractions were collected, desalted and used for LC-MS/MS.

LC-MS/MS and data analyses

LC-MS/MS analyses were performed on a DionexUltimate 3000 n-RSLC system connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Peptides were loaded onto a C18 pre-column (3 μm, Acclaim, 75 μm x 20 mm, Dionex), washed for 3 min at a flow rate of 6 μL/min. Subsequently, peptides were separated on a C18 analytical column (2 μm, Acclaim PepMap RSLC, 75 μm x 25 cm, Dionex) at 350 μL/min via a linear 120-min gradient with HPLC buffer A (0.1 % FA in water) and 25 % HPLC buffer B (0.1 % formic acid in ACN), followed by a 60 min Gradient from 25 to 50% of buffer B. The LC system was operated with Chromeleon Software (version 6.8, Dionex) embedded in Xcalibur software (version 2.1, Thermo Scientific). The effluent from the column was electro-sprayed (Pico Tip Emitter Needles, New Objectives) into the mass spectrometer. The mass spectrometer was controlled by Xcalibur software and operated in the data-dependent mode allowing the automatic selection of a maximum of ten doubly and triply charged peptides and their subsequent fragmentation. A dynamic exclusion allowed up to three repeats. Peptide fragmentation was carried out using High Collision Dissociation settings optimized for iTRAQ-labeled peptides. MS/MS raw data files were processed via Proteome Discoverer 1.4.1.14 mediated searches against UniProtKB/Swiss-Prot protein database (release 0.73).
The following search parameters were used: enzyme, trypsin; maximum missed cleavages, 1; fixed modifications, iTRAQ 4-plex (K), iTRAQ (N terminus), Methylthio (C); variable modifications, oxidation (M), phosphorylation of S, T and Y, respectively; peptide tolerance, 5 ppm; MS/MS tolerance, 100 mnm. All raw data files are uploaded and available at the Pride database.

### Quantitative real-time PCR (qRT-PCR)

RNA was purified from primary cells using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions followed by cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and the anchored-oligo(dT)18 primer. Subsequently, quantitative real-time PCR (qRT-PCR) was performed using the LightCycler 480 SYBR Green I Master Kit (Roche) according to the manufacturer’s protocol and the LightCycler 480 device. Ribosomal protein S9 (RPS9) served as internal reference. Primer sequences are as follows: Temhis forward: GAG CTC CAG TTG GTG TTT TCC AAG GAG TT, RPS9 forward: CTG GAC GAG GGC AAG ATG AAG C and RPS9 reverse: TGA CGT TGG CGG GTT TTC AGA. Temhis reverse: TTG ATG TTT TTC AGT TTT GAA GTGCTTGCGGCT, cloned first into the pENTR™/D-TOPO® vector (Invitrogen) and subsequently into MSCV IRES-Thy1.1 [75] via Gateway® cloning (Invitrogen). CD4^+CD25^+ Treg cells were ex vivo isolated from Foxp3^+CD25^+ reporter mice, stimulated with plate-bound anti-CD3 and anti-CD28 plus 50 ng/μg IL-2 for 40 h, and spin-infected with retrovirus-containing supernatant from either PhoenixEco or HEK293T packaging cells transfected before further analyses.

### Retroviral transductions

Full-length coding sequence of murine Themis was amplified using the primers CACCATGGCTTTATCTCTGGAAGA and TCACAGTG-GAG TT, Themis reverse: TTG ATG TTT TTC AGT TTT GAA GTGCTTGCGGCT, cloned first into the pENTR™/D-TOPO® vector (Invitrogen) and subsequently into MSCV IRES-Thy1.1 [75] via Gateway® cloning (Invitrogen). CD4^+CD25^+ Treg cells were ex vivo isolated from Foxp3^+CD25^+ reporter mice, stimulated with plate-bound anti-CD3 and anti-CD28 plus 50 ng/μg IL-2 for 40 h, and spin-infected with retrovirus-containing supernatant from either PhoenixEco or HEK293T packaging cells transfected with empty or Themis-encoding plasmids (30–70% transfection efficacy). Seventy hours post transduction cells were collected and resorted for transduced Treg cells (CD4^+Foxp3^+CD25^+Thy1.1^+) before further analyses.

### In vitro suppression assay

Naïve CD4^+ T cells (Foxp3^+CD25^-CD62L^high^) were ex vivo isolated from Foxp3^+CD25^+ reporter mice by FACS as described above. Sorted cells were labelled with the amine-reactive dye Cell Trace™ Violet (CTV, Thermofisher Scientific). CTV-labelled naïve T cells were co-cultured with transduced resorted Treg cells (CD4^+Foxp3^+CD25^-Thy1.1^+) at indicated ratios in presence anti-CD3/anti-CD28 coated beads (Life Technologies; ratio of 1:2 beads to cells). After days, proliferation of naïve T cells was assessed by flow cytometry (LSR Fortessa, BD Biosciences) to determine CTV dilution in live naïve T cells, and samples were analysed using FlowJo software (Tree Star Inc).

### Analyses of subcellular TCR signalling components by MELC

Cell preparation and BT-pair formation were performed as previously described [76]. Naïve splenic B cells from BALB/c mice were enriched to over 90% purity by negative isolation using immunomagnetic depletion (Miltenyi Biotech), loaded with 100 μg/ml OVA253-339 peptide for 24 h and washed. Freshly isolated Treg cells and Tconv cells from DO11.10 mice were then mixed at a 1:1 ratio with B cells. To synchronize pair formation, cells were centrifuged shortly at 260 X g and incubated at 37°C and 5% CO₂. At indicated time points BT-pairs were fixed with 2% paraformaldehyde for 15 min and adhered to poly-L-Lysine-coated glass slides. MELC was performed as previously described [21] with an adapted workflow for the analysis of BT-pairs [22]. In brief, antibodies directed against known TCR signalling pathway components (see above) were incubated consecutively and 3D images of the fluorescence signal were acquired by a DM IRE2 microscope (Leica) equipped with a 63x/NA1.40 lens and a KX4 CCD camera (Apogee Instruments) resulting in 3D image stacks of 1024 × 1024 × 32 voxels (voxel size 286 × 286 × 300 nm³).

### Image-processing

Using the corresponding phase contrast images the fluorescence images were automatically aligned voxel-wise with accuracy of one pixel in all dimensions. Fluorescence images were corrected for illumination faults using flat-field correction before the resolution of the wide field fluorescent image stacks were improved by applying a deconvolution/deblurring algorithm (XCOSM software package), an interface to Computational Optical Sectioning Microscopy algorithms for removing out-of-focus light in 3D image volumes (Washington University St. Louis, MO; www.eesrl.wustl.edu/~preza/xcosm/). The IS was manually defined by employing signals from CD3ε, CD45R, F-actin, pSLP76 and pLAT to record positions and size. For all individual BT-pairs we defined regions of interest (ROI) containing all pixels for the B cell and T cell and calculate the ROI for IS, SMAC and cSMAC as well as regions flanking the SMAC within the B cell or T cell (see Fig. 4A and B, and Supporting Information Fig. 8). For these ROI the mean intensities relative to the mean intensity of all pixels belonging to the entire BT-pair (ROI B cell, IS and T cell together) were computed. Heat maps for all regions display the mean of the relative mean intensities or the mean of ratios of relative mean intensities. The distance between the IS and the uropod is the minimum length between the midpoint of the IS and the centroid of the uropod area (Supporting Information Fig. 8).

### Subcellular localization analyses by epifluorescence and spinning disk microscopy

T and B cell pairs were prepared as described above and fixed at different time points with 4% paraformaldehyde in PBS for 20 min, followed by 5 min permeabilisation with 0.15 % Triton X-100 and blocking using 2 % BSA in PBS containing 0.05 % Tween-20. Antibody incubations were performed in blocking solution and coverslips were mounted in Mowiol. Light microscopy
was performed on an inverted microscope (Axiovert 100TV; Carl Zeiss) using standard epifluorescence illumination (light source HXP120, Zeiss) and 100 × /NA1.4 plan-apochromatic objective. Images were acquired with a back-illuminated, cooled charge-coupled-device camera (CoolSNAP HQ2, Photometrics) driven by Metamorph software (Molecular Devices Corp.).

For confocal spinning disk microscopy, ex vivo isolated Tconv cells and Treg cells from BALB/c mice were seeded on anti-CD3ε (0.1 μg/mL; clone 145-2C11; Biologend) and anti-CD28 (1 μg/mL; clone 37.51; Biologend) coated coverslips by brief centrifugation at 260 × g. At indicated time points, cells were fixed, immunostained and mounted as described above. Confocal spinning disk microscopy was performed on an ECLIPSE Ti-E inverted microscope (Nikon) equipped with an Ultra-VIEW VoX Confocal Imaging System (Perkin Elmer), solid-state diode lasers (Perkin Elmer) and using a 100 × /NA1.4 plan-apochromatic objective. Images were acquired using an OrcaR2 CCD digital camera (Hamamatsu). Data acquisition, analyses and 3D rendering were performed using Volocity software (Perkin Elmer).

Statistical analyses

In order to identify statistically significant regulation of proteins within the mouse data sets, Grubbs' test [77] was applied. For this purpose, log2-regulation factors were considered and Grubbs' test was applied repeatedly, where each time the most extreme outlier was deleted until no outliers were present (p = 0.05). Based on boxplots, outliers were identified as those values that were found outside the region four times as large as the interquartile range. Assuming that the regulation factors follow a normal distribution, roughly 0.7% of such outliers are expected. When more than 0.7% outliers in the boxplots were present, the most extreme values above this 0.7% limit were considered as real outliers.

Whereas p-values for the identification of peptides were obtained from Proteome Discoverer, we additionally aimed to evaluate the quality of regulatory information for phosphorylated peptides characterized in this study. For this purpose, we performed a (symmetric) truncation for each log2-regulation factor to estimate standard deviations (SD) data-dependently. Plots of the truncation values against the estimated standard deviation were generated. Results indicated notable fluctuations for log2-regulation factors of smaller 0.3 mainly due the instability of the maximum likelihood estimation procedure and corresponding phosphopeptides were rejected from SD-value estimation. Estimated SD-values for all individual regulation events with log2-regulation factors of larger than 0.3 were then calculated under the assumption of a normal distribution of regulation events (see Supporting Information Table 2, columns H and I).

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