Cofilin1-driven actin dynamics controls migration of thymocytes and is essential for positive selection in the thymus
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ABSTRACT
Actin dynamics is essential for T-cell development. We show here that cofilin1 is the key molecule for controlling actin filament turnover in this process. Mice with specific depletion of cofilin1 in thymocytes showed increased steady-state levels of actin filaments, and associated alterations in the pattern of thymocyte migration and adhesion. Our data suggest that cofilin1 is controlling oscillatory F-actin changes, a parameter that influences the migration pattern in a 3-D environment. In a collagen matrix, cofilin1 controls the speed and resting intervals of migrating thymocytes. Cofilin1 was not involved in thymocyte proliferation, cell survival, apoptosis or surface receptor trafficking. However, in cofilin1 mutant mice, impaired adhesion and migration resulted in a specific block of thymocyte differentiation from CD4/CD8 double-positive thymocytes towards CD4 and CD8 single-positive cells. Our data suggest that tuning of the dwelling time of thymocytes in the thymic niches is tightly controlled by cofilin1 and essential for positive selection during T-cell differentiation. We describe a novel role of cofilin1 in the physiological context of migration-dependent cell differentiation.

KEY WORDS: Cofilin1, Actin filament turnover, Cell migration, T-cell development

INTRODUCTION
Differentiation of αβ T cells follows a complex sequence of molecular events that take place in spatially defined niches of the thymus. T-cell development comprises several distinct stages, each of which is tightly regulated to generate mature and non-self-reactive T lymphocytes: CD4+/CD8− double-negative (DN) precursors develop into CD4+/CD8+ double-positive (DP) cells, before they finally differentiate into CD4+ single-positive (CD4SP) or CD8+ single-positive (CD8SP) thymocytes. The DN cell population can be further characterised by the expression of CD25 (also known as IL2RA) and CD44 (Ceredig and Rolink, 2002; Godfrey et al., 1993), or functionally by a rearranged T-cell receptor (TCR) β locus. After successful β-chain arrangement and expression of a functional pre-TCR, DN thymocytes rapidly proliferate into DP cells and express markers such as CD69 (Von Boehmer et al., 1999). DP cells next undergo TCR α-chain gene re-arrangement, in order to assemble the final αβ TCR complex, which is required for positive and negative selection and generation of CD4SP or CD8SP thymocytes (Jameson and Bevan, 1998; Sebzda et al., 1999). Only then are SP thymocytes licensed to exit the thymus and to constitute the T-cell compartments of the immune system.

Work from many groups has shown that T-cell development is not simply following an autonomous programme, but rather depends on defined spatial cues provided by cell–cell contacts with epithelial stroma cells in the thymus. In this process, thymocytes were found to follow a distinct migratory path between certain areas in the thymus (Lind et al., 2001; Porritt et al., 2003). In the cortex, CD4+/CD8− thymocytes recognise major histocompatibility complex (MHC) ligands on thymic epithelial cells. After successful positive selection, thymocytes then migrate to the central medullary region, where they undergo negative selection (Anderson and Jenkinson, 2001) and elimination of thymocytes that recognise self-peptide-MHC on dendritic and medullary epithelial cells (Le Borgne et al., 2009). To coordinate this process, the cortical and medullary compartments produce distinct chemokines including SDF-1α (also known as CXCL12) and CCL25 (Ladi et al., 2006; Love and Bhandoola, 2011; Takahama, 2006), and provide integrin-mediated adhesion through LFA1 (also known as ITGAL) and ICAM-1 (Fine and Kruisbeek, 1991).

Mutations affecting chemokine signalling or disturbing adhesion can lead to severe changes in thymocyte development (Lancaster et al., 2018; Savino et al., 2004; Uehara et al., 2002a,b). Interestingly, many of these pathways are located upstream of cytoskeletal dynamics. For example, small GTPases are components of the main signalling pathways to cytoskeletal dynamics and have been shown to regulate thymocyte development (Cleverley et al., 1999; Galandrini et al., 1997). Rac-1 specifically has been shown to influence positive and negative selection (Gomez et al., 2001), and WASP was shown to control thymocyte development (Zhang et al., 2002).

Furthermore, T-cell activation through the TCR requires a scaffold of actin filaments (Bunnell et al., 2001; Dustin and Cooper, 2000; Monks et al., 1998; Samstag et al., 2003). Actin polymerisation was shown to be required for T-cell polarization (Delon et al., 1998; Stradal et al., 2006), the formation of the contact zone (Sechi and Wehland, 2004), and T-cell activation via calcium flux, IL-2 production and proliferation (Penninger and Crabtree, 1999). Cell adhesion through integrins was shown to be stabilised by the actin cytoskeleton (Brakebusch and Fässler, 2003). Actin nucleation-promoting factors such as the Abi/WAVE complex or Ena/Vasp can control TCR-mediated actin dynamics (Krause et al., 2000; Nolz et al., 2006; Zipfel et al., 2006). In conclusion, mutations in regulators of the cytoskeleton were shown to cause profound defects in lymphocyte function (Billadeau et al., 2007).

However, little is known about the actual mechanisms by which actin-binding proteins translate the signals into T-cell function. To tackle this question, we need to focus on the downstream effector proteins that directly induce actin filament growth and shrinking. In this context, the F-actin depolymerising proteins of the actin-
depolymerising factor (ADF; also known as DSTN)/cofilin family are among the best-studied molecules. ADF/cofilin members control the on- and off-rate of actin monomers from the filament ends (Carlier et al., 1997), and preferentially sever old (ADP-rich) actin filaments (Maciver et al., 1991; McGough et al., 1997). In mouse and human, three genes – cofilin1, cofilin2 and ADF – are encoded in the genome. They differ in their activities, expression pattern and biological functions (Nakashima et al., 2005; Vartiainen, 2002). ADF/cofilin members were shown to control motility in cultured fibroblast and carcinoma cells (Ghosh et al., 2004; Kato et al., 2008), as well as in human CD4T cells and Jurkat cells (Nishita et al., 2005; Xu et al., 2012).

Systemic deletion of cofilin1 in the mouse was shown to be embryonic lethal (Gurniak et al., 2005), and a cofilin2 mutation resulted in a severe myopathy phenotype (Gurniak et al., 2014), while ADF-deficient mice were viable (Bellenchi et al., 2007). These genetic studies indicated that cofilin1 cannot be functionally replaced by the other two ADF/cofilin members. Indeed, in macrophages, chemotaxis, adhesion and antigen presentation were shown to depend on cofilin1 (Jönsson et al., 2012; Matsu et al., 2001), and, in mature T cells, cofilin1 was shown to provide a link between co-stimulation and the concomitant rearrangement of the actin cytoskeleton (Lee et al., 2000; Samstag et al., 2003). Cofilin1 has been studied with respect to T-cell activation; however, its function in the classical differentiation of α/β T cells in the thymus has been addressed only recently. It was shown that expression of a mutated cofilin1 protein can block T-cell differentiation at the DN stage (Seeland et al., 2018).

Here, we show a new function of cofilin1 as a pacemaker of thymocyte migration and T-cell differentiation in the thymus. Our data identify the turnover of actin filaments as a central mechanism in T-cell development. We also present a mechanism by which cofilin1-controlled resting cycles can affect cell–cell interactions essential for the differentiation of DP thymocytes into mature SP T cells.

RESULTS
Cofilin1 controls actin polymerisation cycles and steady-state levels of F-actin in thymocytes

The aim of our experimental strategy was to genetically interfere with actin dynamics in developing thymocytes by slowing down actin filament turnover. We have shown previously that among the three cofilin/ADF members, cofilin1 is the predominant actin filament-severing factor in haematopoietic cell types (Jönsson et al., 2012). Using isoform-specific antibodies for ADF, cofilin1 and cofilin2, we confirm here that only cofilin1 was expressed in thymocytes, whereas ADF and cofilin2 could not be detected (Fig. 1A). In thymocytes, cofilin1 expression was found to be developmentally regulated (Fig. 1B). While expressed at very low levels in CD4/CD8 DN thymocytes, cofilin1 expression increases in CD4/CD8DP thymocytes, and reaches its highest expression in CD4SP or CD8SP thymocytes. Interestingly, total actin levels were found to fluctuate significantly during thymocyte differentiation, resulting in the highest cofilin1/actin ratio in DP thymocytes. Deletion of cofilin1 in thymocytes should therefore be an ideal tool to blunt actin dynamics in T-cell development in vivo.

To achieve this, we crossed mice carrying a cofilin1 floxed (fl) allele (Gurniak et al., 2005) (Fig. 1C) to a CD4-cre transgenic mouse line expressing cre-recombinase in early thymocytes starting at the DN3 stage (Wolfer et al., 2001). Using a Rosa26-Stop-YFP reporter line (Srinivas et al., 2001), we confirmed CD4-cre mediated deletion already at the DN stage (22% deletion), which then increased to 83% in DP cells and finally reached 99% in CD4SP thymocytes (Fig. 1D). In agreement with the YFP-reporter gene activation, the fl allele was deleted with high efficiency in thymocytes of Cofil1fl/fl,CD4cre mice (Fig. 1E). In total thymocytes, recombination of the fl had occurred with an efficiency of more than 95% (A) as judged by Southern blot analysis. This analysis also confirmed the specificity of cofilin1 deletion in thymocytes, but not in other tissues (Fig. 1E). Concomitantly, cofilin1 protein was depleted in Cofil1fl/fl,CD4cre total thymocytes by more than 90%, as shown by western blot analyses (Fig. 1F).

Importantly, the deletion of cofilin1 did not result in any compensatory upregulation of ADF, cofilin2 or other related F-actin-binding proteins, such as, for example, gelsolin or CapG (Fig. 1F). With the depletion of cofilin1, we consequently removed all ADF/cofilin activity from DP thymocytes. Actin filament dynamics should therefore be severely tuned down. To formally test this assumption, we isolated primary thymocytes from Cofil1fl/fl,CD4cre mice and measured their F-actin and G-actin levels, using fluorescently labelled phalloidin as a sensor for F-actin and fluorescently labelled DNaseI to detect G-actin. Quantitation by fluorescence-activated cell sorting (FACS) analysis revealed a 2.5-fold increase in F-actin levels compared to control thymocytes (Fig. 2A). The increase in F-actin was paralleled by a reduction in the G-actin pool. Quantitation of F- or G-actin levels by fluorescence is very sensitive, but will only allow the measurement of relative changes. To obtain an estimate of the absolute F-actin and G-actin levels, we biochemically separated the Triton X-100-insoluble (F-actin) and -soluble (G-actin) actin pool and determined their amounts by western blotting (Watts and Howard, 1993). In agreement with the FACS data, the biochemical fractionation showed an increase in F-actin and decrease in G-actin in cofilin1-depleted thymocytes (Fig. 2B). Considering the absolute actin levels, the mutant thymocytes still contained ∼60% G-actin. In comparison, control thymocytes contained ∼80% G-actin (Fig. 2B). Although cofilin1-depleted thymocytes still had significant G-actin levels, one important question was whether this monomeric actin pool would still be polymerisation competent upon cell activation. Therefore, we monitored the actual kinetics of actin polymerisation and depolymerisation after stimulation of thymocytes with the chemokine SDF-1α (Fig. 2C). With this assay, we were able to resolve actin polymerisation within seconds and to monitor cycles of F-actin formation over longer periods. Cycling of F-actin levels in stimulated cells is a general phenomenon that was described some time ago (McRobbie and Newell, 1983). In our hands, control thymocytes showed an oscillatory pattern of F-actin levels, with an initial peak around 5 s after stimulation, followed by a second slower rise within the next 30 s and a subsequent return to baseline levels within 1–2 min. For cofilin1-depleted thymocytes, a number of interesting differences were observed: first, thymocytes lacking cofilin1 could polymerise actin upon stimulation, and the F-actin peak increased within 10 s. Second, the initial F-actin increase was significantly exaggerated in cofilin1-depleted thymocytes compared to control thymocytes. Third, the increased F-actin levels of Cofil1fl/fl,CD4cre thymocytes never returned to baseline, even beyond the 5-min end point of the experiment (Fig. 2C). These findings suggest that cofilin1 function is essential to limit the activity of the actin polymerisation machinery. Without cofilin1 activity, actin filament dynamics becomes more refractory to stimulation.

Taken together, we developed and validated a genetic model system that allows the blocking of actin filament dynamics specifically at the DP stage of thymocyte development in vivo. We next focused on our main question of how actin dynamics is linked to thymocyte development.
Cofilin1 is essential for positive selection in the thymus and for the transition of DP cells to CD4SP and CD8SP cells

In the thymus, precursor cells pass several checkpoints on the way to functional T cells. The critical steps are characterised by the sequential expression of the TCR-associated co-receptors CD4 and CD8. First, immature DN thymocytes proceed to the DP stage, which represents close to 90% of thymocytes. In the final differentiation step, DP cells differentiate towards SP cells, which express either CD4 or CD8. Mature T cells can then exit the thymus and home to peripheral secondary lymphatic organs.

In Cof1fl/fl,CD4cre mice, the total number of thymocytes was unchanged (Fig. 3A), suggesting that the deletion of cofilin1 did not have any major adverse effect on proliferation or survival of thymocytes. However, we observed a severe differentiation block from DP cells to SP cells. FACS analysis showed that the thymus of Cof1fl/fl,CD4cre mice was practically devoid of CD4SP and CD8SP thymocytes (Fig. 3B). Since DN and DP cells were not affected in Cof1fl/fl,CD4cre mice, the differentiation block must occur at the transition from DP to SP cells (Fig. 3C). Lineage commitment to CD4SP and CD8SP cells occurs at the late stage of positive selection of DP thymocytes that only survive if they receive a productive signal through their re-arranged TCR (Swat et al., 1993; Yamashita et al., 1993). Cells that have been successfully selected express high levels of the TCRβ-chain (TCRβhigh) and the activation marker CD69 (CD69+). In Cof1fl/fl,CD4cre thymocytes, we found a dramatic reduction in the TCRβhigh/CD69+ subpopulation (Fig. 3D),
suggesting that cofilin1 is critical for the processes involved in positive selection.

The lack of SP thymocytes was further illustrated by the anatomy of the thymus from Cof1fl/Δ,CD4cre mice (Fig. 3E). The area of the cortex was expanded at the expense of the medullary compartment, while the overall size of the thymus was unchanged. The medullary structures were nearly absent in cofilin1 mutant mice; only small fragmented residual islands could be identified (Fig. 3E). The reduction of the medullary compartment is in agreement with the loss of CD4SP and CD8SP thymocytes, since SP thymocytes were shown to support the maintenance of the medulla as a niche (Negishi et al., 1995; Philpott et al., 1992).

Only mature SP T cells will exit the thymus and home into secondary lymphatic organs. However, despite the block in thymocyte development we found a small number of SP T cells in secondary lymphatic organs of Cof1fl/Δ,CD4cre mutants and control mice (Fig. 4A,B). This raised the question on the nature of these T cells that reached ~10% of normal T-cell counts. Were these cofilin1-deleted T cells that had possibly left the thymus?

Expression analyses clarified that the peripheral T cells in Cof1fl/Δ,CD4cre mice were not deleted for the cofilin1 gene and expressed cofilin1 protein. This was illustrated by western blot (Fig. 4C) as well as Southern blot (Fig. 4D) analyses of CD4-sorted peripheral T cells from Cof1fl/Δ,CD4cre mice. The rationale to use Cof1fl/Δ,CD4cre mice for this experiment was to better illustrate the lack of deletion by the equal signal intensities of fl and Δ alleles. If complete deletion had occurred, the fl signal should have disappeared; if partial deletion had occurred, the fl signal should have diminished and the Δ allele increased. The equal ratio of the fl and Δ alleles indicated that none of the fl alleles had been converted to the Δ allele and that practically all the T cells found in the periphery had escaped cofilin1 gene deletion (Fig. 4D).

The presence of functional escape T cells in the periphery also showed that the differentiation block is a cell-autonomous phenomenon of mutant thymocytes and not caused by alterations in the thymic stroma environment. These data show that T cells can still emerge from a morphologically altered thymus as long as they express cofilin1. The low abundance of T cells in secondary lymphatic organs of Cof1fl/Δ,CD4cre mice (Fig. 4B) did not affect the B-cell representation as shown by CD19-positive cells in lymph node as an example (Fig. 4E). One should note that the apparent increase in B cells in the FACS plot is owed to the representation of relative numbers of T cells and B cells. The absolute numbers of B cells in lymph nodes of mutant and control mice are in fact not significantly altered (Fig. S1E).

**Thymocyte proliferation and apoptosis are independent of cofilin1**

We next addressed the underlying mechanisms responsible for the differentiation block and the severe reduction of T cells in Cof1fl/Δ,CD4cre mutants. One trivial explanation could simply be a block in thymocyte proliferation and/or increased cell death.

The total cell counts in the thymus of Cof1fl/Δ,CD4cre mutants and control mice were identical, as well as the pools of DN and DP thymocytes (see Fig. 3A,C). This suggested that alterations in the proliferation and apoptosis equilibrium were unlikely to account for the block in thymocyte development. Nonetheless, we quantitated apoptosis in isolated total thymocytes as well as DP cells, using active caspase 3-specific antibodies. We did not observe any significant difference between control mice and Cof1fl/Δ,CD4cre mutant mice (Fig. 5A,B). In steady-state conditions, apoptotic cells in the thymus are cleared very rapidly and therefore it might be difficult to detect small changes in apoptotic numbers. To uncover a possibly mild phenotype we challenged isolated total thymocytes in vitro with phorbol myristate acetate (PMA)/ionomycin as growth stimulators. Both control thymocytes and cofilin1-depleted thymocytes showed comparable cell expansion in vitro (Fig. 5C).

To address the proliferation potential, we cultured total thymocytes in the presence of phorbol myristate acetate (PMA)/ionomycin as growth stimulators. Both control thymocytes and cofilin1-depleted thymocytes showed comparable cell expansion in vitro (Fig. 5C). In addition, cell cycle analysis ruled out any defect in cytokinesis, as indicated by the normal cell distribution in G0/G1- and G2/M-phases (Fig. 5D).
One critical parameter in T-cell development is the sequential turnover of surface receptors such as CD4 and CD8, which are crucial for T-cell function. In thymocytes, cofilin1 is not required for surface receptor trafficking. It was previously shown that cofilin1 deficiency promotes apoptosis in thymocytes and cells did not respond to SDF-1 chemokine. Similarly, cofilin1-deficient thymocytes did not respond to the chemokine. Without stimulation, as well as in the presence of SDF-1α, adhesion to ICAM-1 was strongly impaired in cofilin1-depleted thymocytes, whereas cofilin1-deficient thymocytes did not respond to the chemokine. Similar results were obtained when we measured thymocyte spreading on ICAM-1-coated surfaces. Spreading goes hand in hand with adhesion and strictly depends on the re-organisation of the actin cytoskeleton (Brakebusch and Fässler, 1993). As seen for adhesion, spreading was impaired in cofilin1-deficient thymocytes and cells did not respond to SDF-1α (Fig. 6B). It is noteworthy that the levels of ICAM-1 receptors were not changed in cofilin1-deficient thymocytes, as shown by FACS analysis of total thymocytes from control (dashed lines) and Cof1fl/fl,CD4cre mutant mice. In summary, our data show that cofilin1 deficiency does not promote apoptosis in thymocytes and nor does it affect cell proliferation.

In thymocytes, cofilin1 is not required for surface receptor turnover

One critical parameter in T-cell development is the sequential up- and downregulation of surface receptors such as CD4 and CD8, as well as in softer 3-D substrates. A transwell assay was performed to address chemotaxis in surface-dependent motility, whereas a collagen matrix was used to investigate migration in 3-D. In both

Cofilin1 is controlling cell adhesion and 3-D migration of thymocytes

DP thymocytes mature along a migratory path through the thymus. On the way through the cortex and the medulla, thymocytes receive signals via cell–cell contacts that are essential for their differentiation. Consequently, cell adhesion is a critical parameter in thymocyte development. For example, the interaction of thymocyte LFA-1 with epithelial cell ICAM-1 is one important pathway for T-cell differentiation (Fine and Kruisbeek, 1991; Lub et al., 1997). Chemokines such as SDF-1α and CCL25, both produced by stromal cells, play a pivotal role in integrin-mediated thymocyte adhesion and development (Nornent and Bevan, 2000; Peled et al., 2000; Uehara et al., 2002a).

We therefore tested whether cofilin1 deficiency affects thymocyte adhesion to ICAM-1. Without stimulation, as well as in the presence of SDF-1α, adhesion to ICAM-1 was strongly impaired in cofilin1-depleted thymocytes (Fig. 6A). In addition, SDF-1α stimulation could augment the percentage of adhering control thymocytes, whereas cofilin1-deficient thymocytes did not respond to the chemokine. Similar results were obtained when we measured thymocyte spreading on ICAM-1-coated surfaces. Spreading goes hand in hand with adhesion and strictly depends on the re-organisation of the actin cytoskeleton (Brakebusch and Fässler, 2003; Stossel, 1993). As seen for adhesion, spreading was impaired in cofilin1-deficient thymocytes and cells did not respond to SDF-1α (Fig. 6B). It is noteworthy that the levels of ICAM-1 receptors were not changed in cofilin1-deficient thymocytes, as shown by FACS analysis of LFA-1 subunit chains CD11a and CD18 (Fig. S1A).

Adhesion and spreading are crucial for cell migration. Most cells go through cycles of attaching, spreading and leading-edge protrusion, followed by cell rounding and pausing before another migration cycle is re-initiated (Friedl et al., 2001). Having identified cofilin1 as a parameter for thymocyte spreading and adhesion, we wanted to directly address cofilin1 function in thymocyte migration. To this aim, we employed two conceptually different migration assays, which allowed us to dissect cell motility on hard surfaces as well as in softer 3-D substrates. A transwell assay was performed to address chemotaxis in surface-dependent motility, whereas a collagen matrix was used to investigate migration in 3-D. In both

Collectively, these data show that cofilin1 deficiency does not promote apoptosis in thymocytes and nor does it affect cell proliferation.
assays, we used SDF-1α and CCL25, the two most abundant chemokines in the thymic cortex, to stimulate migration (Wurbel et al., 2000; Zaitseva et al., 1998).

Unstimulated migration of cofilin1-deficient thymocytes was significantly reduced in the transwell assay (Fig. 6C). Likewise, chemotaxis towards the applied chemokines was impaired, despite the fact that cofilin1-depleted thymocytes showed increased migration compared to medium control (Fig. 6C). We again validated that the respective chemokine receptors for SDF-1α (CD184; also known as CXCR4) and CCL25 (CD199; also known as CCR9) were indeed from CD4SP and CD8SP T cells in the periphery of Cof1fl/fl,CD4cre mice. (B) Quantitation of peripheral T cells (lymph node, spleen, blood) with respect to total lymphocytes in control (ctrl.) and Cof1fl/fl,CD4cre mice. Note that Cof1fl/fl,CD4cre mice are shown to directly illustrate the ratio of escaper cells in the periphery. The equal intensity of the fl and Δ alleles indicates that none of the fl alleles was converted to the Δ allele and that the T cells found in the periphery had escaped cofilin1 gene deletion. (C) Western blot of CD4-sorted T cells from spleen confirmed normal expression levels of cofilin1 protein in peripheral T cells. Equal numbers of CD4-sorted cells from control (ctrl.) and Cof1fl/fl,CD4cre mice were loaded, and the blot was probed with a cofilin1-specific antibody. (D) Southern blot analysis of genomic DNA from CD4-sorted T cells, showing that the T cells found in the periphery had escaped cofilin1 gene deletion. (E) FACS analysis of B- and T-cell ratio in lymph nodes using CD19 as a marker for B cells and TCRβ as a marker for T cells (n=5).

In conclusion, our results provide new insights into the role of cofilin1 in 3-D cell migration. Cofilin1 is controlling adhesion, the pattern of migration cycles and the average velocity of thymocytes. Together, these very specific alterations result in the failure of thymocyte development in vivo.

Thymocyte differentiation can be uncoupled from cofilin1-dependent migration in vitro

Our results strongly argue for a cofilin1-dependent ‘motility pattern’ as the determining parameter for thymocyte differentiation in vivo. One prediction would then be that all cell-inherent differentiation parameters, such as signalling mechanisms or gene expression patterns, should function independently of cofilin1. If this was true, we should be able to circumvent the migration-dependent differentiation block by providing the relevant external signals in vitro. This should then allow cofilin1-depleted DP thymocytes to differentiate towards SP thymocytes.

To address this final hypothesis and test the uncoupling of differentiation from migration, we set up an ex vivo differentiation experiment. It was shown that incubation of DP thymocytes with a...
A mix of TCRβ and CD2 antibodies can mimic positive selection and push thymocyte differentiation predominantly towards CD4SP cells (Cibotti et al., 1997). Following this strategy, we stimulated thymocytes for 32 h in culture and tested the conversion of DP towards SP cells. In our hands, more than 50% of control DP thymocytes differentiated into CD4SP cells (Fig. 7A). Similarly, more than 50% of Cof1fl/fl,CD4cre thymocytes converted to CD4SP cells (Fig. 7B). The same result was obtained when in vitro differentiation was triggered by an increase in intracellular calcium via PMA/Ionomycin treatment (data not shown) (Mitnacht et al., 1998; Ohoka et al., 1996). These data demonstrated that cofilin1-deficient DP thymocytes have the full capacity to differentiate into SP cells.

In summary, our data demonstrate that cofilin1 is central to the regulation of a ‘stop-and-go’ mode of thymocyte migration and adhesion, which in a physiological context appears to be essential for providing environmental cues and cell interactions necessary for thymocyte differentiation.

**DISCUSSION**

Imaging experiments performed in the thymus suggested that thymocyte migration is an important parameter for differentiation. For example, thymocytes depend on environmental cues provided by cell–cell contacts with epithelial stroma cells (Takahama, 2006), and during positive selection thymocytes migrate with defined pausing cycles that allow interaction with MHC-expressing epithelial cells (Sebzda et al., 1999).

However, it has remained unclear how these specific aspects of thymocyte motility are tuned on the level of the actin cytoskeleton.

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**Fig. 5.** Apoptosis, cell proliferation and receptor transport in thymocytes is cofilin1 independent.

(A) Intracellular staining for active caspase 3 in total thymocytes and DP thymocytes. FACS analysis of control (ctrl.) and Cof1fl/CD4cre thymocytes showed comparable percentages of apoptotic cells (n=3; s.d. is shown with n.s.P<0.5; unpaired two-tailed Student’s t-test). (B) DP thymocytes kept in culture for 0 h and 4 h were analysed by FACS for the percentages of Annexin V-positive cells (n=5; s.e. is shown with n.s.P<0.5; unpaired two-tailed Student’s t-test). Note that prolonged culture conditions increase the number of apoptotic cells equally in controls and Cof1fl/CD4cre thymocytes. (C) Proliferation of total thymocytes after PMA/Ionomycin stimulation (+PMA/I) and without stimulation (–PMA/I). Under both conditions control thymocytes (black lines) and Cof1fl/CD4cre thymocytes (grey lines) showed comparable expansion (n=8; s.e. is shown with n.s.P<0.5; unpaired two-tailed Student’s t-test). (D) Cell cycle analysis of total thymocytes. DNA content was quantified by FACS analysis after staining cells with propidium iodide. The percentages of cells at different cell cycle stages were similar in control (ctrl.) and Cof1fl/CD4cre thymocytes. (E) CD4 and CD8 surface expression on thymocytes was determined by FACS analysis before (untreated) and 14 h after Pronase treatment at 4°C or 37°C. Recovery of CD4/CD8 expression is shown in the upper-right quadrant, and is comparable at 37°C in control and Cof1fl/CD4cre thymocytes. (F) Kinetics of CD4/CD8 receptor recovery from 0 h to 14 h after Pronase treatment in control (ctrl., solid line) and Cof1fl/CD4cre thymocytes (dotted line) (n=5, s.d. is shown with n.s.0.05<P; unpaired two-tailed Student’s t-test).
Signalling pathways controlling cytoskeletal dynamics have been identified, and mutations in WASP (Zhang et al., 2002), Rac-1 (Gomez et al., 2001) and other small GTPase pathways have suggested a link to actin (Costello et al., 2000). It is, however, difficult to unequivocally link these data to actin dynamics, as all of the mentioned targets entertain a crosstalk with numerous other cellular processes that might very well influence thymocyte differentiation, independent of actin regulation.

We therefore developed strategies to study the direct link of actin filament dynamics to thymocyte migration and differentiation. It was our objective to downregulate actin filament turnover specifically in thymocytes, without interfering with any upstream signalling pathways or any other second cell type. Key to this strategy was to blunt all actin filament-depolymerising activity by deleting cofilin1.

Cofilin1 is a member of a family of actin-depolymerising factors and the only ADF/cofilin protein expressed in thymocytes. Previous work from our group has identified cofilin1 as the central actin filament severing activity in vivo (Flynn et al., 2012; Jönsson et al., 2012). Related actin filament-severing proteins such as gelsolin are not relevant for the present study, since gelsolin is expressed at very low levels, and previous work had excluded any important role in T-cell development (Witke et al., 1995).

Cell type-specific deletion of cofilin1 therefore provided an excellent tool to address actin filament dynamics in thymocyte development. We generated a conditional Cof1fl/fl,CD4cre mouse model, in which cofilin1 gene deletion occurred with high efficiency in early DP T-cell precursors in the thymus. It is an intrinsic property to the hematopoietic system that even high cre-recombinase expression rates of more than 99% will allow a small...
number of cells to escape recombination and hence maintain expression of the target gene. In this study, we indeed observed a small percentage of ‘escaper cells’, which expressed cofilin1 and developed normally into mature CD4 and CD8 T cells. This escaper fraction in fact served as a convenient control for supporting the cell-autonomous function of cofilin1 in thymocytes (see below).

Upon deletion of cofilin1, neither ADF nor cofilin2 expression was found to compensate its absence in thymocytes. In agreement with the overall loss of severing activity, we observed a pronounced increase in the F-actin content in thymocytes. Interestingly, the mutant thymocytes were still able to trigger actin polymerisation upon stimulation, suggesting that the mechanisms of actin nucleation and polymerisation can work independently of cofilin1, as long as a sufficient pool of polymerisation-competent G-actin is available. In this context, it is important to note that G-actin pools can be very different, depending on the cell type. For example, classical adherent cells such as fibroblasts have roughly 50% F-actin and 50% G-actin (Phillips et al., 1980). In resting thymocytes, on the other hand, we found that only 20% of total actin is in the filamentous form, leaving 80% as monomeric G-actin pool. Despite the F-actin increase in cofilin1-deficient thymocytes, this still leaves ~60% of cellular actin to the G-actin pool. More important than steady-state levels of F- or G-actin were the dynamic changes in F-actin, namely the actin polymerisation and depolymerisation kinetics. When cofilin1 was deleted, stimulation of actin polymerisation in thymocytes resulted in overshooting F-actin levels, which did not return to baseline levels during the timeframe of the experiment. Therefore, one major function of cofilin1 appears to be to counteract the polymerisation burst in a feedback loop, which ultimately allows an oscillating mode of actin polymerisation and depolymerisation. It is important to note that, in our experiments, we followed global actin polymerisation in the cell.

What are oscillatory F-actin fluctuations good for, and what is their possible role in a physiological context? Thymocytes have to migrate through the thymic epithelial tissue in order to sense local chemokines and interact with stromal signals to differentiate into mature T lymphocytes (Savino et al., 2004). Two photon studies on thymic tissue have shown that thymocytes exhibit mainly random walking through the cortex until they undergo selection, which appears to trigger a rapid directed migration towards the medulla (Witt et al., 2005). This implies that a sophisticated modulation of migration patterns is a major parameter of thymocyte differentiation.

With this in mind, we can picture cofilin1 function in the thymus. Without cofilin1, DP thymocytes are developmentally arrested and do not differentiate into CD4SP and CD8SP thymocytes. Our data show that this phenomenon is associated with the lack of F-actin oscillations, which in turn cause an unusual ‘motility pattern’ of cofilin1-deficient thymocytes. In a 3-D matrix this is illustrated by the prolonged pausing and lower migratory speed of Cof1fl/fl,CD4cre thymocytes. In addition, integrin-mediated cell adhesion and spreading of thymocytes is dependent on cofilin1. The combined migration and adhesion defects we observed ex vivo, however, become critical in vivo in the tissue context. Apparently, cofilin1 is required for stirring thymocytes through the thymus and to allow cell-cell contacts to occur in a coordinated fashion. All our data presented here are in good agreement with this hypothesis. Cofilin1-depleted thymocytes miss these cues, but can be forced to differentiate into SP thymocytes simply by activating the respective signalling pathways in vitro. This demonstrated that cofilin1-deficient thymocytes still have the intrinsic capacity to differentiate once the appropriate signals are provided.

Our data also excluded a number of rather trivial explanations for the observed differentiation block. Previously described functions of cofilin1 in apoptosis (Chua et al., 2003; Wabnitz et al., 2010), cell proliferation (Eibert et al., 2004; Ohashi, 2015) and receptor trafficking (Salvarezza et al., 2009; von Blume et al., 2011) seem to be rather cell-specific functions, but not relevant in thymocyte development. In addition, it is important to note that the function of cofilin1 is clearly cell autonomous in thymocytes. As mentioned, our CD4-cre mouse model allows a small percentage of thymocytes to escape deletion. These escaper cells express cofilin1, developed into mature CD4SP or CD8SP cells, and populated peripheral lymphatic tissues. This finding excludes epiphenomena due to tissue architecture or any other secondary effects. In addition, unpublished data from our group on the deletion of cofilin1 in mTECs using a K14-cre driver line, showed no impairment in thymocyte development (C.G. and W.W., unpublished).

In Cof1fl/fl,CD4cre mutant mice, the block of αβ thymocyte differentiation had a number of physiological consequences, such as the severe lack of peripheral T cells and the relative increase of B cells in secondary lymphatic compartments. Alterations in the B cell compartments had been observed before (Molina et al., 1992; Negishi et al., 1995); however, in our Cof1fl/fl,CD4cre mutant mice the absolute number of B cells in secondary lymphatic tissues was unchanged (Fig. S1E). In this context we should note that heterozygous cofilin1 mutants (Cof1fl/fl,CD4cre), expressing 50% of normal cofilin1 protein levels showed no impairment in T-cell development or alterations in any T-cell subset we analysed.

Control of cofilin1 activity is a powerful means of regulating T-cell differentiation. In the physiological context, cofilin1 is regulated by different mechanisms (Bernstein and Bamburg, 2010; Van Troys et al., 2008). One control mechanism is phosphorylation of Ser3, which is shutting off activity while dephosphorylation activates cofilin1 (Nagaoka et al., 1996). Studies have shown dephosphorylation of cofilin1 after T-cell stimulation (Samstag et al., 1992); however, whether phosphorylation is relevant in thymocytes during the initial F-actin burst has not yet been addressed. In a recent study, it was shown that expression of a GFP-cofilin1 variant in DN cells that cannot be phosphorylated is blocking T-cell development at the DN stage (Seeland et al., 2018). In this model, the GFP-cofilin1 variant apparently has a dominant blocking T-cell development at the DN stage (Seeland et al., 2018). In this model, the GFP-cofilin1 variant apparently has a dominant blocking T-cell development at the DN stage (Seeland et al., 2018).
cells or other cell types (Eibert et al., 2004; Lee et al., 2000). Our approach does indeed not allow us to draw any conclusions on cofilin1 function in mature T cells upon stimulation and cell activity.

In conclusion, we propose a new function of cofilin1 in regulating the coordinated migration and adhesion of thymocytes during positive selection and differentiation into SP T cells.

MATERIALS AND METHODS

Mouse lines

The generation of the cofilin1 conditional mouse line (Cof1^floxfloxCdes+) was described by Gurniak et al. (2005) and the respective alleles are illustrated in Fig. 1C. Cof1^floxfloxCdes+ mice were crossed to a transgenic mouse line, which expresses Cre recombinase under the CD4 promoter (Wolfier et al., 2001). Animals homozygous for the cofilin1 fl allele and heterozygous for the CD4-cre allele (Cof1^floxfloxCdes+) were used for experiments. Unless specified differently, control mice were either littermates homozygous for the cofilin1 flox allele or mice wild type for cofilin1 and heterozygous for CD4-cre (Cof1^floxflox+/Cdes+). The Rosa26-Stop-YFP reporter line carries a flexed ‘Stop cassette’ in the ubiquitously expressed Rosa26 locus (Sinivas et al., 2001). Upon Cre-mediated deletion of the ‘Stop cassette’, YFP expression can be observed. Unless specified otherwise, the n given in the figure legends refers to the biological n, meaning the number of animals used in independent experiments. Genotyping was performed by PCR using gene- and mutation-specific primers. Animal housing, breeding and procedures were carried out according to EU regulations, and permission was granted by local authorities in Italy and Germany (Decretto n.19/2005-B and AZ 84-02.04.2017:A088).

Cell preparation and sorting

Tissues were obtained from adult 6-week- to 3-month-old animals and single-cell suspensions were prepared from thymus in RPMI 1640 medium without Phenol Red. For Southern and western blot analyses of peripheral T cells, CD4+ cells from spleen were sorted using magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). As judged by FACS staining according to the protocol of Friedl et al. (1998) and Quast et al. (1997), 90% pure.

Flow cytometry

Up to four-colour FACS staining was performed as described by Gurniak and Witke (2007). Briefly, single-cell suspensions were filtered (30 µm Milcons) and stained with the corresponding antibodies in FACS buffer [0.5% fetal calf serum (FCS), 2 mM EDTA in PBS] for 15–30 min on ice, washed two times with FACS buffer and data were acquired on an Accuri C6 [Becton Dickinson (BD)]. For actin measurements, to determine G- and F-actin levels by DNase and phalloidin staining, cells were fixed in 4% paraformaldehyde (PFA)/PBS, permeabilised for 15 min with 0.1% Triton X-100/PBS, with both incubations at room temperature. For apoptosis staining, for apoptotic and dead cells, an Annexin V-FITC Apoptosis Detection Kit (BD) was used in combination with propidium iodide, and staining for CD4-PerCP and CD8-allophycocyanin (APC) was performed simultaneously. For intracellular staining of active caspase 3, live cells were first stained for CD4 and CD8, then fixed with 4% paraformaldehyde (PFA)/PBS, permeabilised by 0.1% Triton X-100/PBS, and stained with Alexa Fluor 488-conjugated anti-active caspase 3 (Cell Signaling Technology). Incubation steps with fluorophores were carried out on ice, and reagents were diluted in FACS buffer. For fluorescent labelling, we used the following antibodies from eBioscience at 1:100 dilution: FITC-conjugated anti-CD4 (cat. no. 11-0042-85), anti-CD8 (cat. no. 11-0081-86) and anti-TCRβ (cat. no. 11-5961-85); APC-conjugated anti-CD4 (cat. no. 11-5961-85); anti-CD8 (cat. no. 17-0081-83); anti-CD184 (cat. no. 17-991-80); anti-CD199 (cat. no. 17-991-80); and phycoerythrin (PE)-conjugated anti-CD69 (cat. no. 12-0691-83) and anti-TCRβ (cat. no. 12-5961-83). We also used the following reagents from BD at 1:100 dilution: FITC-conjugated anti-CD8 (cat. no. 553031); phycoerythrin (PE)-conjugated anti-CD19 (cat. no. 557399); PerCP-conjugated anti-CD19 (cat. no. 551001) and anti-CD8 (cat. no. 553036); PerCPfluor710®-conjugated anti-CD4 (cat. no. 553052); APC-conjugated anti-CD19 (cat. no. 550992); and 7-aminoactinomycin D (7-AAD). Reagents used from Molecular Probes® were Alexa Fluor 680-conjugated phalloidin and Alexa Fluor 488-conjugated DNSel.

Thymocyte culture and treatments

For in vitro differentiation of thymocytes as described by Cibotti et al. (1997), 3 cm polystyrene Petri dishes (Greiner Bio-One) were coated for 3 h at room temperature with anti-TCRβ and anti-CD2 antibodies (eBioscience) at a concentration of 5 µg/ml in 50 mM Tris-HCl pH 9.5. Then, 1×10^7 thymocytes were cultured at 37°C, 5% CO2 in 1 ml RPMI 1640 medium containing 1.76 µM 2-mercaptoethanol and 10% heat-inactivated FCS, which had been depleted of endogenous steroids by charcoal and dextran. After 18 h, cells were washed and transferred for recovery culture onto uncoated Petri dishes for an additional 16 h, before FACS analysis. For pronase treatment, the CD4/CD8 receptor re-expression assay was essentially performed as described previously (Ohoka et al., 1996; Punt et al., 1996). Briefly, thymocytes were washed three times with PBS and 2×10^6 cells/ml were treated two times with 0.02% Pronase (Calbiochem) and 100 µg/ml DNSel (Promega) diluted in PBS for 15 min at 37°C. To quench the reaction, 10% FCS was added and cells were washed three times with RPMI 1640 medium. For receptor recovery, cells were incubated in RPMI 1640 medium for 1, 6 and 14 h at 37°C, 5% CO2 or at 4°C, before staining and FACS analysis.

Cell cycle analysis and proliferation assay

The cell cycle kit by BD was used to analyse the DNA content of total thymocytes. After fixation, RNase treatment and propidium iodide staining, single cells were acquired by flow cytometry. To determine the proliferation rate of total thymocytes, 5×10^4 cells were seeded into 96-well plates and incubated for 0, 24, 48 and 72 h with or without 10 ng/ml PMA and 400 nM ionomycin in RPMI 1640 medium at 37°C, 5% CO2. After centrifugation and removal of medium, plates were frozen at −80°C. Cell number was determined by staining of DNA with Cyquant™ (Thermo Fisher Scientific) and fluorometric analysis.

Adhesion, spreading and migration assays

The adhesion assay on ICAM-1 was performed as outlined by Quast et al. (2009) and Boehm et al. (2003). For efficient coating of ICAM-1 to the plastic surface, a precoating of human IgG Fcγ monoclonal antibody (Dianova) followed by binding of recombinant mouse ICAM-1-Fc chimera (R&D Systems) was used. As a control, we kept an ICAM-1-uncoupled ring area on the plastic surface. Thymocytes were stimulated with 400 ng/ml SDF-1α (PeproTech) for 60 min and cells were then allowed to attach for 1 h. Unbound cells were rinsed off with Hank’s balanced salt solution until the outer uncoated ring area was devoid of cells. Adherent cells were then fixed with 4% PFA/PBS for 10 min and counted manually in seven random fields of view using a 10×-phase-contrast objective (Nikon) with a fully automated inverted microscope (Keyence). To determine spreading, the number of extended cells were counted from phase-contrast images. Statistics were performed by two-tailed independent Student’s-t test and one-way ANOVA using SigmaPlot® 12 (Systat Software, Inc.).

The 2-D migration of thymocytes was measured by transwell migration using inserts with a pore size of 5 µm (Corning Costar), as described by Phee et al. (2010) and Shioi et al. (2008). Single-cell suspensions were preincubated for 1 h in migration medium [10 mM HEPES pH 7.2, 100 U/ml penicillin/streptomycin, 0.5% (v/v) BSA in RPMI 1640] at 37°C, 5% CO2 and then 3×10^5 cells were transferred to the top chamber of the respective transwell plates. Assays were set up in duplicate; the bottom chamber contained plain migration medium or one of the chemokines – SDF-1α (400 ng/ml) or CCL25 (2.5 µg/ml) (both from PeproTech) – diluted in migration medium. After an incubation time of 5 h at 37°C, 5% CO2 cells were collected from the bottom well and stained with CD4-PerCP, CD8-PE and TCRβ-APC antibodies and analysed by flow cytometry. The receptors for the chemokines SDF-1α and CCL25 were stained with the antibodies CD184-APC and CD199-APC, respectively.

For the analysis of thymocyte motility in 3-D, migration assays were performed in polymerised rat tail collagen-I (Gibco® INVITROGEN) according to the protocol of Friedl et al. (1998) and Quast et al. (2009). On ice, collagen-I was titrated with 0.1 N NaOH and mixed with 10× minimum essential medium (Gibco® INVITROGEN) and the chemoattractant SDF-1α.
(400 ng/ml) or CCL25 (2.5 μg/ml) (PeproTech). Then, 3×10^5 thymocytes in migration medium were carefully mixed with 100 μl collagen solution, resulting in a final collagen-I concentration of 3 mg/ml. Collagen-thymocyte mixtures were placed in custom-built chemotaxis chambers and incubated for 60 min at 37°C, 5% CO2; to allow collagen polymerisation and cell recovery. Time-lapse imaging was conducted using a fully automated inverted microscope (Keyence) equipped with a 10× phase-contrast objective (Nikon) and a climate chamber (37°C, 5% CO2). Time-lapse images were recorded for 8 h at 90 s intervals; control and mutant thymocytes were simultaneously acquired at different locations. ImageJ 1.47t software (NIH) and the ‘Manual Tracking’ plug-in were used to track cell paths. The Chemotaxis and Migration Tool Version 1.01 plug-in (ibidi Integrated BioDiagnostics) was employed for plotting cell tracks and for the measurement of velocity and distance. Movies were processed using ImageJ 1.47t. Statistics were performed by SigmaPlot® 12 by Mann–Whitney test and two-tailed independent Student’s t-test.

**Histology**

Haematoxylin and Eosin (H&E) staining was carried out on 8 μm cryosections; after fixation with 4% PFA/PBS slides were incubated in 0.05% Eosin (Sigma-Aldrich) and 20% Haematoxylin (Merek Millipore). Area measurements of thymic regions were performed manually on H&E-stained sections using the area measurement plug-in of the Keyence BZ-II analyser software (version 1.41).

**Western blot analysis**

Total protein lysates were prepared from single cell suspensions boiled in SDS sample buffer (22 mM Tris-HCl, pH 6.8, 4% glycerol, 0.8% SDS, 1.6% 2-mercaptoethanol, Bromophenol Blue). The samples were separated by SDS-PAGE, transferred to Immobilon-P membrane (Merck Millipore) and probed with antibodies. Signals were developed using enhanced chemiluminescence and recorded digitally on a LAS4000 (General Electric). Equal loading was verified by Coomassie Blue staining with GAPDH as an internal standard. Antibodies used were mouse anti-GAPDH (Merek); rabbit anti-cofilin1, rabbit anti-cofilin2 mouse anti-ADF (Gurniak et al., 2014); rabbit anti-gelsolin (Witke et al., 1995); rabbit anti-CapG (Witke et al., 2001); anti-actin (MP Biomedicals); horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-rat IgG (Jackson ImmunoResearch).

**Southern blotting**

CD4-cre mediated deletion of the floxed cofilin1 alleles was analysed by Southern blotting (Witke et al., 2001); anti-actin (MP Biomedicals); horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-rat IgG (Jackson ImmunoResearch).


