The chaperonin CCT8 controls proteostasis essential for T cell maturation, selection, and function

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T cells rely for their development and function on the correct folding and turnover of proteins generated in response to a broad range of molecular cues. In the absence of the eukaryotic type II chaperonin complex, CCT, T cell activation induced changes in the proteome are compromised including the formation of nuclear actin filaments and the formation of a normal cell stress response. Consequently, thymocyte maturation and selection, and T cell homeostatic maintenance and receptor-mediated activation are severely impaired. In the absence of CCT-controlled protein folding, Th2 polarization diverges from normal differentiation with paradoxical continued IFN-γ expression. As a result, CCT-deficient T cells fail to generate an efficient immune protection against helminths as they are unable to sustain a coordinated recruitment of the innate and adaptive immune systems. These findings thus demonstrate that normal T cell biology is critically dependent on CCT-controlled proteostasis and that its absence is incompatible with protective immunity.

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Cells are indispensable co-ordinators of the adaptive immune response and embrace different effector functions dependent on the context in which they recognize their cognate antigen\textsuperscript{1}. The competence of T cells to respond adequately to antigenic challenges is inextricably linked to de novo protein expression and a change in the cell’s protein homeostasis. Under optimal conditions, this process relies on a complex network of interconnected, dynamic systems that control protein biosynthesis, folding, translocation, assembly, disassembly and clearance\textsuperscript{2}. Cell stress and other physiological demands on the cell’s proteome can however result in challenges where nascent and metastable proteins are misfolded and, as a result, aggregate-entrapped polypeptides are formed. These conformational changes, as toxic intermediates, a cell’s functions and may impair its survival. Thus, protein quality control and the maintenance of proteostasis are essential for almost all biological processes. This is in part accomplished by a machinery of chaperones that catalytically resolve misfolded proteins from adopting a state of amorphous aggregates but assist them in assuming a native conformation\textsuperscript{3,4}. Chaperones are operationally defined by their capacity to interact transitorily with other proteins in assisting de novo folding of nascent proteins, refolding of stress-denatured proteins, oligomeric assembly, protein trafficking and proteolytic degradation\textsuperscript{5}. Chaperones that partake in de novo folding or refolding promote these conformational changes through the recognition of hydrophobic amino acid side chains of non-native polypeptides and proteins. Eukaryotic type II chaperones, aka chaperonins, are large (~800–900 kDa), cytoplasmic, protein complexes (designated CCT, chaperonin containing tailless complex polypeptide 1, TCP-1 or TCP-1-ring complex, TRIC) that are built as hetero-octameric cylinders formed from two stacked doughnut-like rings\textsuperscript{6}. Each of the rings is composed of homologous, yet distinct 60 kDa subunits (α, β, γ, δ, ε, ζ, η and θ)\textsuperscript{7}; CCTs recognize, bind and globally enclose protein substrates of up to ~60 kDa to allow their folding over several cycles. Multiple substrates are recognized by CCTs whereby each of the complex’s subunits may identify different polar and hydrophobic motives\textsuperscript{8}. High-affinity substrates are evicted from CCTs in an ATP-dependent fashion as they act as competitive inhibitors of the complex’s catalytic reaction of unfolding proteins\textsuperscript{9}.

As many as 10% of newly synthesized proteins are assisted by CCT to adopt a correct conformation\textsuperscript{10}, including key regulators of cell growth and differentiation, and components of the cytoskeleton\textsuperscript{11,12}. Actins and tubulins have been identified as two of the major folding substrates of CCT\textsuperscript{13}. Adequate production of effector cytokines by T cells has been related to rapid actin polymerization and the generation of a dynamic filament network in the nucleus of CD4\textsuperscript{+} T cells\textsuperscript{14}. Moreover, signal transduction, cytoskeletal synthesis and remodelling, immune synapse formation, macromolecular transport and cell division require molecules that depend on CCT function\textsuperscript{15,16}.

To dissect the precise role of CCT in T cell development and function, we generated mice that lack the expression of a single subunit, CCTθ (aka CCT8), in immature thymocytes and their progeny. This particular subunit was chosen as previous investigations had identified CCT8 to be upregulated in activated and polarized T cells\textsuperscript{17} and to be a key regulator in assembling the TrIC complex\textsuperscript{18}. We tested the proficiency of these cells to develop normally, be selected within the thymus, and respond to antigens as part of an adaptive immune response. Our results show that CCT8 is largely, albeit not entirely, dispensable for thymocyte differentiation and selection but essential for mature T cells to respond adequately to antigenic stimuli.

**Results**

**Thymocyte development and peripheral T cell differentiation depend on CCT8 expression.** The subunit 8 of type II chaperones, CCT8, was detected throughout thymocyte development but was most prominently found in immature cells with a double negative (DN, i.e. CD4–CD8–) phenotype (Fig. 1a). CD4-Cre: CCT8fl/fl mice (designated CCT8\textsuperscript{−/−}; Supplementary Fig. 1 and Supplementary Data 1 and 2) have a loss of CCT8 expression targeted to double positive (DP i.e. CD4\textsuperscript{+} and CD8\textsuperscript{+}) thymocytes and their progeny (Supplementary Fig. 1). The total thymus cellularity of these mice was comparable to that of Cre-negative littermates (designated CCT8\textsuperscript{+/+}; Fig. 1b). As expected, the frequency of DN and immature single positive (ISPDC8) thymocytes remained unaffected as the deletion of CCT8 occurs after these maturational stages (Fig. 1c and Supplementary Fig. 1). However, the frequency of DP thymocytes was mildly increased and their progression to a post-signalling stage (TCR\textsuperscript{β}CD69\textsuperscript{+}) was impaired (Fig. 1d), which correlated with a higher number of thymocytes that had not received a sufficiently strong survival signal (Fig. 1e). In parallel, the extent of negative selection during the first selection wave, as identified by the co-expression of Helios and PD1 on Foxp3–CCR7–TCR\textsuperscript{β} DP or CD4 thymocytes (aka wave 1a and b, respectively), was reduced in CCT8\textsuperscript{−/−} mice (Fig. 1f, g). The following wave, which takes place in the medulla and is characterized by Helios expression on Foxp3–SPDC8 thymocytes, was reduced in a first (CD24\textsuperscript{−}) but not a second phase (CD24\textsuperscript{+}; Fig. 1h, i). Finally, fewer phenotypically and functionally mature single positive CD4\textsuperscript{+} (SP4) and SPDC8 thymocytes were detected at a late stage of their development (Fig. 1c, j, k and Supplementary Fig. 1) and the frequencies of thymic and recirculating regulatory T cells (Treg) were likewise reduced (Fig. 1l, m). Hence, the targeted loss of CCT8 expression in thymocytes impaired their selection and reduced the frequency of post-selection, mature effector and regulatory T cells.

The total splenic cellularity of CCT8\textsuperscript{−/−} mice was normal, although drastically fewer naive T cells were detected (Fig. 2a, b and Supplementary Data 1 and 2) and the CD4 and CD8 lineages were differentially affected (Fig. 2c). The frequencies of CD4 and CD8 T cells with a memory phenotype were increased (Fig. 2d), likely reflecting homeostatic expansion as a consequence of low T cellularity. Correspondingly, the frequency of peripheral Treg cells (CD25\textsuperscript{+}FoxP3\textsuperscript{+}) remained unaffected in CCT8\textsuperscript{T−/+} mice but the subpopulation of highly suppressive CD103\textsuperscript{+}ICOS\textsuperscript{+} Treg was several fold increased in line with the extent of lymphopenia (Fig. 2e, f)\textsuperscript{19}. Moreover, the frequency of CD4\textsuperscript{+} memory T cells with an anergic phenotype (CD73\textsuperscript{+}FR4\textsuperscript{−}) was reduced in CCT8\textsuperscript{−/−} mice (Fig. 2g). Collectively and contrary to the relatively minor decrease in thymic SP cells, the loss of CCT8 expression correlated with a severe reduction in peripheral T cells implying a functional impairment of these cells.

**Loss of CCT8 impairs the formation of nuclear actin filaments.** To assess the proliferative capacity of T cells deficient or proficient for the expression of CCT8, naive CD4\textsuperscript{+} T cells were labelled with the membrane dye cell trace violet, activated (using cross-linking with anti-CD3 and anti-CD28 antibodies) and cultured for a total of 96 h. Activated CCT8\textsuperscript{T−/+} T cells displayed a drastically reduced expansion index when compared to controls and their survival was greatly reduced, which could not be enhanced by the addition of IL-2 or the anti-oxidant N-Acetyl-L-cysteine (Fig. 3a, Supplementary Fig. 2, Supplementary Table 1 and Supplementary Data 1 and 2)\textsuperscript{20}. Because T cell activation is associated with significant de novo protein synthesis\textsuperscript{21} and the engineered lack of CCT8 expression reduced the expression of all components of the CCT complex (Fig. 3b), we next quantified in
CCT8-deficient and -proficient T cells changes in tubulin and actin expression, as these two serve as folding substrates for the CCT complex. Several tubulin isoforms were reduced in CCT8−/− T cells, independent of the activation state, while the detection of the ubiquitously expressed β and γ actin isoforms was unaffected by a loss of normal CCT expression (Fig. 3c and Supplementary Data 3). However, the formation of nuclear actin filaments could only be detected in a very small fraction of CCT8-deficient T cells (3.17 ± 1.15% versus 29.40 ± 10.5%) similar to the frequency of wild-type T cells in which Arp2/3 was pharmacologically inhibited and actin nucleation was prevented (Fig. 3d, e). Hence, the formation of nuclear actin filaments, an
**Fig. 1 The role of CCT8 in thymocytes.** a) CCT8 protein detection by western blot. Values shown are relative to the detection in DN thymocytes. b) Total thymic cellularity, and c) thymocyte subpopulations in 6-week-old CCT8<sup>T<+/+</sup> (grey bars) and CCT8<sup>T<–/–</sup> mice (white bars) as defined by CD4 and CD8 cell surface expression on lineage-negative thymocytes. d) Positive thymocyte selection and maturation stages (R2: TCR<sup>β+</sup>CD69<sup>hi</sup>CCR7<sup>−</sup>SPCD4<sup>−</sup>SPCD8<sup>−</sup> and R5: TCR<sup>β+</sup>CD69<sup>−</sup>), and progression between sequential stages. e) Non-signalled thymocytes (activated caspase 3<sup>+</sup> expression among CD5<sup>−</sup>TCR<sup>β+</sup> thymocytes). Negative thymocyte selection in the cortex. f) Wave 1a (TCR<sup>β+</sup>hiCCR7<sup>−</sup>SPCD4<sup>−</sup>SPCD8<sup>−</sup>), and g) wave 1b (TCR<sup>β+</sup>SPCD4<sup>−</sup>CCR7<sup>hi</sup>CD69<sup>−</sup>CD24<sup>−</sup>). Negative selection in the medulla. h) Wave 2a (TCR<sup>β+</sup>SPCD4<sup>−</sup>CCR7<sup>−</sup>CD69<sup>−</sup>CD24<sup>+</sup>), i) and wave 2b (TCR<sup>β+</sup>SPCD4<sup>−</sup>CCR7<sup>hi</sup>CD69<sup>−</sup>CD24<sup>−</sup>). j) Late-stage maturation of single positive TCR<sup>β+</sup> thymocyte subpopulations in 6-week-old CCT8T<sup>T<+/+</sup>CCT8T<sup>T<–/–</sup> mice (white bars). Gating strategies are shown for representative contour plots.

**Fig. 2 Peripheral T cell cellularity and phenotype in the absence of CCT8 expression.** Analysis of 4–6-week-old CCT8<sup>T<+/+</sup> (grey bars) and CCT8<sup>T<–/–</sup> mice (white bars). Gating strategies are shown for representative contour plots. a) Total splenic cellularity. b) Frequencies of splenic B and T cells. Frequencies (c) of total splenic CD4 and CD8 T cells, and (d) their naive (CD62L<sup>+</sup>CD44<sup>−</sup>) and memory, effector memory (CD62L<sup>−</sup>CD44<sup>−</sup>) and central memory subpopulations (CD62L<sup>−</sup>CD44<sup>+</sup>), respectively. e) The frequencies of total splenic T<sub>reg</sub> cells, and f) T<sub>reg</sub> cell subpopulations defined by ICOS and CD103 cell surface expression. g) Frequency of anergic CD4<sup>+</sup> T cells in lymph nodes. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Student’s t-test, (a–g)). Contour plots (b–g) are representative of data in bar graphs. Data shown in bar graphs represent mean ± SD values of a single experiment and are illustrative of two independent experiments with three replicates each. See also Fig. S1.
important requirement for T cell function, was dependent on an intact CCT complex.

The lack of CCT8 compromises proteostasis in both resting and activated T cells. Upon T cell activation, the lack of CCT8 also changed the expression of proteins other than individual CCT components and tubulin (Fig. 3f and Supplementary Data 3). At least six different patterns of protein changes were detected when comparing mutant and wild-type T cells before and 24 h after activation, including proteins that were minimally expressed in both resting and activated CCT8−/− T cells (groups b, c).
I+II), or that could be detected in unstimulated CCT8T<sup>+/+</sup> T cells but following activation did not change (groups III–V) or even a decrease in expression was detected (VI) (Fig. 3f). Many of the group I proteins were related to mitochondrial functions, for example, the ATP synthase membrane subunit f (Atp5j2, catalysing ATP synthesis<sup>24</sup>), glutamate dehydrogenase 1 (Gld1, catalysing the oxidative deamination of glutamate<sup>25</sup>) and prohbitin (Phb, controlling mitochondrial biogenesis and also cell-cycle progression, nuclear transcription and resistance to various apoptotic stimuli)<sup>26,27</sup>. CCT8T<sup>+/+</sup> CD4 T cells showed increased expression of mitochondrial-associated proteins and genes relative to CCT8T<sup>+/−</sup> CD4 T cells, which was in agreement with the cells’ inadequate ability to increase protein synthesis and to adapt to metabolic demands (Fig. 3g, h and Supplementary Fig. 2). This deficiency in mitochondrial-related pathways was a consistent finding in both the proteome and transcriptome (Fig. 3g, h and Supplementary Fig. 2), and particularly focused on an altered expression of coenzyme Q10 metabolism and ATP synthesis. However, neither the biogenesis nor the membrane potential of mitochondria were impaired in activated T cells (Supplementary Fig. 2). We noticed in resting T cells a reduction in the concentration of Arpc1b (group II) and a lack of an upregulation of Arpc4 (group VI) in activated T cells, representing two of the five essential and non-interchangeable components of the Arp2/3 complex which promotes filamentous (F) actin branching (see Fig. 3d)<sup>28</sup>. The organizer proteins Moesin (Msn) and Ezrin (Ezr) (Group III) that link F-actin to the plasma membrane remained highly expressed in stimulated CCT8T<sup>+/−</sup> T cells thus impairing the remodelling of the cytoskeleton upon activation<sup>29</sup>. Furthermore, several proteins failed to be reduced in response to CD4<sup>+</sup> T cell activation (group III) including Ifit1, a protein expressed in response to interferons and the subsequent recruitment of STAT1 that also negatively regulates pro-inflammatory genes<sup>30</sup>. Moreover, flow cytometry confirmed that STAT1 and its activated form, phospho-STAT1 (pSTAT1) were increased both before and 24 h after mitogenic stimulation (Fig. 3i). RNA-Seq analysis showed differential gene expression and confirmed for activated CCT8T<sup>+/−</sup> T cells an enrichment of genes belonging to the IFN-γ pathway (GO:0034344; 8.8-fold change in mutant when compared to wild-type cells, adjusted p-value = 0.0002; Supplementary Fig. 2)<sup>31</sup>.

CCT8 is essential to avert T cell activation-induced cellular stress. The accumulation of unfolded protein in the endoplasmic reticulum (ER) leads to cellular stress and, where unresolved, a loss of regular cell functions prompting apoptosis. Eukaryotic cells have developed an evolutionary well-conserved mechanism to clear unfolded proteins and to restore ER homeostasis, known as the unfolded protein response (UPR)<sup>32</sup>. The UPR comprises a tightly orchestrated collection of signalling events that are controlled by protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring protein-1α (IRE-1α), which collectively sense ER stress and alleviate the accumulation of misfolded proteins, for example, via increasing the expression of ER chaperones<sup>33</sup> (Supplementary Fig. 3).

Naive CD4<sup>+</sup> and CD8<sup>+</sup> T cells from CCT8<sup>+/−</sup> and CCT8<sup>+/+</sup> mice, respectively, were activated for 48 h using anti-CD3 and anti-CD28 antibodies to detect UPR-related cell stress. Activated wild-type CD4<sup>+</sup> T cells exposed to Tunicamycin to induce ER stress were used as a positive control. Increased transcripts for Perk, Atf6 and Ire1-a, were detected in activated CCT8<sup>+/−</sup> CD4<sup>+</sup> T cells when compared to controls (Fig. 4a). In keeping with and consequent to an activated UPR, untreated CCT8<sup>+/−</sup> CD4<sup>+</sup> T cells and treated CCT8<sup>+/+</sup> CD4<sup>+</sup> T cells increased their transcripts for the chaperone glucose-regulated protein (Glp) 78, a target gene of ATF6, and XBP-1 which is placed in the ER lumen and contributes there to ER homeostasis, protein folding and degradation<sup>33,34</sup>. Activation of Ire1-a also led to an upregulation of pro-apoptotic Bim and a decreased expression of the anti-apoptotic Bcl2, thus contributing to the impaired survival of activated CCT8<sup>+/−</sup> CD4<sup>+</sup> T cells (Fig. 4a, Supplementary Data 1). In contrast, CCT8<sup>+/−</sup> CD8<sup>+</sup> T cells displayed only a limited upregulation of Perk expression, whereas components of the other ER stress pathways were either unchanged or even diminished when compared to wild-type CD8<sup>+</sup> T cells. Taken together, activated CD4<sup>+</sup> + CCT8<sup>+/−</sup> T cells displayed an extensive UPR as a result of impaired proteostasis, which appeared incompatible with normal T cell function.

**Th2 cell polarization and T cell metabolism are dependent on CCT8 expression.** Activated T cells undergo clonal expansion and differentiate, in the presence of additional molecular cues, into functionally distinct T subsets characterized by separate cytokine profiles and effector behaviours. Because mitochondrial and proteomic reprogramming parallel this differentiation, we next examined whether an inadequate mitochondrial response to metabolic demands, combined with an abnormal UPR (Supplementary Fig. 3), could impair peripheral T cell differentiation. Under Th1 polarizing conditions, the absence of CCT8 expression did not impair the viability of in vitro activated CD4 T cells but increased their frequency to express the signature cytokine IFN-γ (Fig. 4b and Supplemental Data 1 and 2). In contrast, both viability and IL-4 production were reduced in activated CD4 CCT8<sup>+/−</sup> T cells polarized to adopt a Th2 phenotype (Fig. 4c). Paradoxically, the frequency of IFN-γ expressing CD4 CCT8<sup>+/−</sup> T cells was increased by 7-fold under these conditions. Both of these findings are in agreement with an increased detection of STAT1 and pSTAT1 in CCT8<sup>+/−</sup> T cells, independent of their activation status (Fig. 3e). Conditions favouring T<sub>reg</sub> differentiation not only caused a reduced viability in CCT8<sup>+/−</sup> T cells, but also resulted in a reduced conversion of these cells to express FoxP3, whereas the vast majority of viable cells unexpectedly expressed IFN-γ (Fig. 4d). Driving CCT8<sup>+/−</sup> T cells to a Th17 phenotype correlated with a dramatic loss in viability albeit the
Fig. 4 Peripheral T cell functions in the absence of CCT8 expression. Analysis of naive CD4+ T cells from 4-6-week-old CCT8T+/+ (grey bars) and CCT8T−/− mice (white bars). a qPCR analysis of ER stress response elements in CD4+ and CD8+ T cells activated by CD3 and CD28 cross-linking and cultured in the presence or absence of Tunicamycin (CD4+ cells only); expression normalized to GPDH and displayed as 2−ΔΔCT values relative to values from CCT8T+/+ CD4+ and CD8+ T cells arbitrarily set 1. b In vitro differentiation of peripheral naive CD4+ T cells grown for 5 days under differentiating conditions: frequency of live cells (left) and cells (right) adopting: b Th1 polarization; c Th2 polarization; d Treg differentiation; and e Th17 differentiation. f Uptake of fatty acids in CD4+CD62L+CD44− (naive) and CD62L−CD44+ (memory) cells ex vivo activated by CD3 and CD28 cross-linking for 20 h. g Expression of long-chain fatty acid receptor CD36 on CD3/CD28-activated cells, and h uptake of glucose analogue 6-NBDG in CD4+CD62L+CD44− (naive) and CD62L−CD44+ (memory) cells. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data were calculated by ANOVA correcting for multiple comparisons (Holm-Sidak method) (a) and Student’s t-test (b-h). Bar graphs show mean ± SD and are representative of two independent experiments with three replicates each. See also Fig. S3.
frequency (but not the total cellularity) of living cells successfully polarized was increased (Fig. 4e). This reaction was CCT8\(^{-/-}\)-T cell-intrinsic, as a comparable response was observed when CCT8-deficient and -proficient T cells were co-cultured during polarization (Supplementary Fig. 3). Thus, polarization of T cells was severely altered in the absence of CCT8, thus favouring these cells to adopt a Th1 phenotype.

As T cells transform from a quiescent to an activated state, the generation of energy from shared fuel inputs such as fatty acids and glucose are essential for the cells’ growth, differentiation and survival\(^3,4\). We therefore tested whether an absence of CCT8 expression impaired the use of these two essential energy sources by CD4\(^+\) T cells. Fatty acid uptake was significantly reduced in both activated naïve and memory CD4\(^+\) T cells lacking CCT8 (Fig. 4f). This result was notably independent of an increased cell surface expression of CD36, a glycoprotein that acts together with chaperones to translocate fatty acids to the cytoplasm (Fig. 4g). In contrast to wild-type controls, both naïve and memory CCT8\(^{+/+}\)-T cells displayed a higher glucose uptake (Fig. 4h) indicating a compensatory mechanism to be in play that secures, at least in part, the cells’ energy expenditure. Indeed, basal respiration, spare respiratory capacity and extracellular acidification rate (ECAR), which measures glycolysis, remained globally normal despite a lack in CCT8 (Supplementary Fig. 3). Hence, the absence of CCT8 in T cells resulted in a change in energy usage which may further explain the impaired response to cell activation.

CCT8 is essential for protective immunity against intestinal helminths. Infection with the nematode *Heligmosomoides polygyrus* activates a strong Th2-type immune response\(^37\). Primary infections in susceptible strains such as C57BL/6 are normally non-resolving and clearance requires drug treatment with an anti-helminthic such as pyrantel embonate. In contrast, spontaneous clearance of secondary infections relies on a protective anti-helminthic immune response requiring the exposure to the parasite\(^42\). We therefore also determined the absolute number and frequency of B cells in the peritoneal lavage of infected wild-type and CCT8\(^{-/-}\)-mice (Fig. 5i) and probed the secretion of *H. polygyrus*-specific serum IgG1. In comparison to infected control mice there was a higher frequency of B cells in the mesenteric lymph nodes of mutant mice but fewer in the peritoneal cavity during both primary and secondary infections (Fig. 5i and Supplementary Fig. 5). In parallel, CCT8\(^{-/-}\)-mice failed to generate an antigen-specific IgG1 response to a secondary *H. polygyrus* infection (Fig. 5f) further highlighting the consequences of a limited Th2 response in vivo.

The type 2 cytokines IL-5 and IL-13 are also secreted early during a helminth infection by innate lymphocytes (ILC2) in response to the release of IL-33 and TSLP from mucosal epithelial sensor cells\(^33,44\). We therefore quantified these innate lymphocytes in mesenteric lymph nodes and in the peritoneal lavage of infected wild-type and CCT8\(^{-/-}\)-mice. During the initial infection, CCT8\(^{-/-}\)-mice had a higher frequency of ILC2 at both anatomical sites but the absolute cellularity was comparable to that of control animals (Fig. 5b, k, l). Upon re-infection with *H. polygyrus*, the ILC2 frequency was comparable for CCT8\(^{-/-}\)- and CCT8\(^{+/+}\)-mice but the absolute cellularity was lower in mutant mice (Fig. 5k, l). This finding correlated with a lack of intestinal tuft cell expansion during secondary infection in mutant mice, suggesting the feed-forward loop, in which type 2 cytokines increases the numbers of these cells\(^44,45\), was not supported by resident ILC2 cells indicating an adaptive T cell contribution is required which is absent in the CCT8\(^{-/-}\)-mice (Supplementary Fig. 4). In addition, the scarcity of AAMacs, eosinophils and B cells in the peritoneal lavage of CCT8\(^{-/-}\)-mice demonstrated that the overall provision of type 2 cytokines in response to the nematode was inadequate to recruit an effective cellular and humoral response to *H. polygyrus*.

**Discussion**

CCT captures and manipulates the folding of non-abundant intermediates from a range of substrates whereby its interaction occurs in a subunit-specific and geometry-dependent fashion\(^46\). We demonstrate here that a targeted loss of only the CCT8 protein compromised the function of the entire CCT complex and thus impaired the correct folding and consequent function of different substrates, including the cytoskeletal proteins actin and tubulin. Actin with its V-shaped molecular structure\(^47\) has been identified as a prototype substrate that requires CCT’s function for its efficient folding\(^46\), a process involving the binding to CCT4 and either CCT2 or CCT5 but spares direct contacts with CCT8\(^48,49\). The biogenesis of actin is, however, unaffected by the depletion of CCT8, a finding in line with observations in *C. elegans* where actin levels remain normal despite an impaired CCT8...
Fig. 5 The response to primary and secondary *H. polygyrus* infection. a Eggs per gram of faeces at indicated timepoints. Comparison of CCT8<sup>T+</sup>/+ (black circles) and CCT8<sup>T−/−</sup> mice (grey squares). b Cellularity in mesenteric lymph nodes, and peritoneal lavage. c Th2 cell frequency in mesenteric lymph nodes, and d peritoneal lavage. e Treg frequencies in mesenteric lymph nodes, and f peritoneal lavage. Frequencies of (g) alternatively activated macrophage and (h) eosinophils in peritoneal lavage. I B cells in peritoneal lavage, and j *H. polygyrus*-specific serum IgG1. ILC2 frequency in mesenteric lymph nodes (k) and peritoneal lavage (l). c-f and k, l Display of contour plots with gating. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data were compared by Student’s t-test adjusted for multiple comparison (Holm-Sidak method). Bar graphs show mean ± SD and are representative of one of the two independent experiments with at least 4 samples per group.
function. In this experimental system, aggregates including actin are not efficiently cleared from the cytoplasm causing the formation of aggregates and misfolded proteins that convey either a loss, a change or even a gain of function as a result of their toxicity.

Productive folding and processing of actin in T cells is an important prerequisite for the signalling-dependent changes in shape. Indeed, T cells adapt to different physiological conditions including, for example, the stress of the bloodstream shape. Indeed, T cells adapt to different physiological conditions important prerequisite for the signalling-dependent changes in toxicity.

The formation of aggregates and misfolded proteins that convey either a toxic effect or a more beneficial role is a common phenomenon in many cell types. These structures play a pleiotropic role in T cell activation as they control the promotion of conjugates, the activation and nuclear import of transcription factors, and, possibly, the internalization of the TCR.

Our results demonstrated that depleting CCT constrains the nuclear formation of actin filaments and results in a panoply of functional changes. For instance, TCR-mediated progression of CCT8-deficient thymocytes past several intrathymic checkpoints was impaired, including the cells’ normal positive and negative selection. These findings align well with studies that have identified a role for UPR in thymocyte maturation and selection (reviewed in ref. 32). Interestingly, the observed partial block in thymocyte maturation was relatively mild despite a complete absence of CCT8 at the DP and later stages of thymocyte maturation. In stark contrast, the periphery of CCT8-/-/- mice is severely lymphopenic indicating peripheral T cells to be especially reliant on this complex for their maintenance and function. Indeed, both ER stress and UPR play an important role in the homeostatic maintenance of peripheral T cells and are disordered in the absence of CCT, especially CD4+ T cells. In contrast, the UPR of CD8+ T cells was minimally affected by the absence of CCT8 for reasons yet to be determined. The mass spectrometric analysis of both resting and activated CCT8-/-/- T cells further confirms at the protein level that several molecules involved in mitochondrial functions, including Phb1 and Phb2, are expressed at lower concentrations in the absence of CCT8. Phb1 and Phb2 form a hetero-oligomeric complex that contributes, inter alia, to an UPR in mitochondria.

In addition to a compromised cell viability and a restricted clonal expansion, the absence of CCT in activated T cells also causes an increased ability to secrete IFN-γ and a limited efficiency to adopt a Th2 phenotype, even when exposed in vitro to ideal polarizing conditions. While Th-bet favours the expression of IFN-γ the same factor represses the Th2 lineage commitment via a tyrosine-kinase-mediated interaction that disrupts the binding of GATA3 to its DNA binding motif. We noted under non-polarizing condition an upregulation of total and activated STAT1 in CCT8-/-/- cells both before and 24 h after activation of primary CD4+ T cells, whereas neither Th-bet nor IL-12 receptor expression were detected. Though previously thought to play a role in STAT1 biosynthesis, CCT is apparently not critically required for the formation of STAT1. The phosphorylation of GATA3 and activation of signal transducer and activator of transcription 5 (STAT5) are two indispensable events for this differentiation process to occur.

The co-evolution of vertebrate hosts with helminths has resulted over the course of millions of years in a sophisticated immune defence that engages several effector mechanisms orchestrated by a robust Th2-type response. This reaction activates and mobilizes a suite of innate immune cells and local tissue responses. During the worm’s life cycle, infective larvae taken up by oral route invade the mucosa of the duodenum, cross its muscle layer and reach a space beneath the serosa from where adult parasites return 8 days later to access the intestinal lumen. Even though a primary infection does not resolve without drug treatment under the experimental conditions used here, a secondary exposure is completely cleared in wild-type mice when sufficient IL-4 (but not necessarily IL-13) is available to activate responsive epithelia and AAMacs forming granulomatous cysts to encase the larvae that have penetrated the intestinal wall.

The activation and expansion of B cells secreting cytokines and immunoglobulins, in particular IgG1 in a T cell-dependent fashion, are known to be critical for effective anti-H. polygyrus immunity. Resistance to H. polygyrus (re-) infection also results in the activation of group 2 innate lymphoid cells (ILC2), which are required for the differentiation of conventional Th2 cells. However, the limited capacity of CCT8-/-/- mice to effectively polarize their CD4+ T cells in vivo to a Th2 phenotype thwarts the clearance of H. polygyrus upon re-infection even in the presence of ILC2 cells. Total cellularity and the frequency of Th2-type T cells are significantly reduced in both mesenteric lymph nodes and the peritoneal lavage of infected CCT8-/-/- mice when compared to wild-type animals. This reduction and its downstream cellular and molecular consequences—possibly in addition with the general lymphopenia of CCT8-/-/- mice—explains mechanistically the animals’ inability to expel H. polygyrus via a robust immune response that generates sufficient IL-4. Interestingly, ILC2 in both mesenteric lymph nodes and the peritoneal lavage increase in CCT8-/-/- mice during the first worm exposure indicate that the initial recruitment of these cells is largely intact. However, these cells require a greatly expanded Th2 population, and subsequent cytokine secretion, to escalate a full-scale anti-helminth immune reaction including tuft cell proliferation and alternative activation of macrophages. Although ILC2 cells are an innate source of IL4, their reduced cellularity is insufficient to create an effective immune defence in CCT8-/-/- mice to bypass their defective Th2 response. This and additional findings presented here indicate that Th2-type cells are indispensable for a protective immunity against H. polygyrus whilst positioned upstream of AAMac, eosinophils and B cells whose activation and expansion cannot be driven by ILC2 alone.

H. polygyrus infections also expand and activate the host’s Threg population, especially early in an infection when these cells appear to outpace the proliferation of effector T cells and modulate the immune response. An increased frequency of Threg possibly as a result of low-dose IL-4 exposure was observed in the mesenteric lymph nodes of infected CCT8-/-/- animals where these cells may further inhibit Th2 immunity. However, the frequency of Threg was significantly reduced in the peritoneal lavage. The molecular reason for this compartmentalization of Threg remains so far unknown, but may reflect a deficiency in the cell’s expression of CD103 and homing to the intestine. This could result from a lack in upregulating the gut-trophic chemo- kinase receptor CCR9 and/or an irregular response to CCL25, a chemokine highly expressed in the epithelium of the inflamed small intestine and on postcapillary venules of the lamina propria.

In mouse strains susceptible to H. polygyrus infections, Th2 responses are counterbalanced by IFN-γ-producing CD4+ and CD8+ T cells. The predilection of CCT8-/-/- T cells to adopt a Th1 phenotype and secrete IFN-γ upon activation suppresses the formation of a type 2 immune response and inhibits cell proliferation, thus further contributing to a deviation away from protective immunity against H. polygyrus. It is tempting to
speculate that such a shift towards a Th1-type immune response due to an absence of regular CCT8 function may be harnessed therapeutically in non-infectious, Th2-driven pathologies (e.g. allergic diseases). However, the benefit of such an imbalance towards a Th1-weighted immune reaction requires further probing, for example, in the context of an anti-viral immune response where IFN-γ discloses both anti-viral and immunomodulatory functions to combat the infection while minimizing collateral tissue damage22.

Taken together, CCT-mediated protein folding is essential for normal T cell biology as their development, selection and function are severely impaired by a lack of normal proteostasis. In this study, we demonstrate for the first time that the loss of CCT function in T cells disturbs normal proteostasis and the dynamic formation of nuclear actin filaments, a prerequisite for normal cell-cycle progression and chromatin organization. As a consequence, the maintenance of a normal peripheral T cell pool is severely compromised which correlates with an inadequate mitochondrial response to metabolic demands and an abnormal UPR which impairs the ability of cytokine stimulation and cell stress. Finally, CCT function is required for T cells to be Th2 polarized and to stage a protective immune response against H. pyloryrus via adequate feed-forward loops engaging multiple cellular effector mechanisms.

Methods

Materials availability. Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author.

Experimental model. Mice. The C57 KO mouse model (Cct8tm1a(KOMP) Wtst, Project ID CSD453801) was obtained from the KOMP Repository (www.komp.org) and generated by the Wellcome Trust Sanger Institute (WTSI). Targeting vectors used were generated by the Wellcome Trust Sanger Institute and the Children’s Hospital Oakland Research Institute, as part of the Knockout Mouse Project (3U01HG004808), designed to delete exon 2 in the Cct8 gene. The Cd4-Cre transgenic mouse was originally developed at the University of Washington on C57BL/6 background23. Mice between 4 and 10 weeks of age were used for experiments. Animals were maintained under specific pathogen-free conditions and experiments were performed according to institutional and UK Home Office regulations.

Methods details. Flow cytometry, cell sorting and cell preparation by magnetic-activated cell separation. Cells from thymus, spleen and lymph nodes were isolated from wild-type and mutant mice and stained using combinations of the antibodies listed in Supplementary Data 4. Where needed, unmanipulated naive CD4+ cells were enriched using magnetic separation (Miltenyi Biotec). Before staining, cells were resuspended at a concentration of 1 × 10^6/100 µL in PBS containing 2% FCS (Merck). Staining using magnetic separation (Milteny Biotec). Before staining, cells were resuspended with 0.1% Saponin. Prior to cytokine staining, cells were incubated with Cell Stimulation Cocktail (eBioscience) for 2 h before adding monensin, and further incubated at 37 °C for a total of 5 h. Cell viability was measured using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific) as per the manufacturer’s instructions. After staining, the cells were acquired and sorted using a FACS Aria III (BD Biosciences) and analysed using FlowJo v10.

Detection of TNF-α production. Assay of TNF-α production was performed in accordance with48. Brefly, total thyomocytes were activated with 1 µg/ml plate-bound anti-CD3 and soluble 1 µg/ml anti-CD28 in the presence of monomins (BioLegend). After 4 h, the cells were stained for surface markers (SM, M1, M2 panel), treated with Foxp3 Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer’s instructions before staining for intracellular TNF-α.

Primary cell culture, activation, proliferation and polarization. Naive CD4+ T cells were labelled by Cell Trace Violet (ThermoFisher Scientific) according to the manufacturer’s protocol and activated in vitro using a combination of plate-bound anti-CD3 (2 µg/ml) (BioLegend) and soluble anti-CD28 (2 µg/ml) (BioLegend) antibodies in RPMI (Merck), containing 10% Heat Inactivated FCS (Invitrogen) and 1% Penilin-Streptomycin (Merck). Cell proliferation was measured as dilution of the cell dye as assessed by Flow cytometry. For in vitro polarization, naive T cells were freshly isolated and subsequently cultured for 4.5 days in RPMI (Merck), containing 10% Heat Inactivated FCS (Invitrogen) and 1% Penilin-Streptomycin (Merck). Following conditions were used: Th1: 50 U/ml IL-2, 2 µg/ml anti-CD28, 3 µg/ml IL-12, 10 µg/ml anti-IL-4; Th2: 50 U/ml IL-2, 2 µg/ml anti-CD28, 1 µg/ml anti-CD3, 10 µg/ml IL-4, 10 µg/ml anti-IFN-γ; Th17: 1 µg/ml anti-CD28, 5 ng/ml TGFβ, 10 ng/ml IL-1β, 50 ng/ml IL-6, 20 ng/ml IL-23, 10 ng/ml anti-IFN-γ, 10 µg/ml anti-IL-4; Treg: 50 U/ml IL-2, 2 µg/ml anti-CD28, 5 ng/ml TGFβ.

Quantitative real-time PCR (qPCR). cDNA was synthesized from total RNA of isolated thyomocytes and T cells, and qPCR performed according to the manufacturer’s instruction (Bioline). All primers (Merck) were designed to span exon–exon boundaries and are available upon request. For the detection of Cct8 transcripts, primers spanning exon 2 was designed. Primer sequences are listed in Supplementary Data 4. The expression of the following genes was used to assess ER stress: Bcl2, Grp78, Atf6, Irela, Perk and Bim. Expression of Gapdh was used as an internal reference, and the delta CT method was used in order to normalize expression. The delta delta CT (ΔΔCT) method was used for analyses of fold change, which was expressed as 2^-ΔΔCT.

Phalloidin staining for nuclear actin filaments. Phalloidin staining for nuclear actin was adapted from49. Briefly, CD 4 T cells were isolated from lymph nodes of C57Bl/6 mice at 4 weeks of age and plated O/1 µg/ml anti-CD1 and 1 µg/ml anti-CD2 at 4 × 10^5 cells/ml in starvation media (RPMI (Merck), containing 0.5% Heat Inactivated FCS (Invitrogen) and 1% Penilin-Streptomycin (Merck)) with wild-type cells added CK666 (Merck), an Arp2/3 inhibitor, as negative control. The next day, cells were collected in 100 µl media (RPMI (Merck), containing 0.5% Heat Inactivated FCS (Invitrogen) and 1% Penilin-Streptomycin (Merck)) with 1 µg/ml phalloidin-5680 FITC (ThermoFisher Scientific) and 5 µg/ml Propidium iodide (ThermoFisher Scientific). The nuclei were then stained with 0.1% Saponin. After 5 h, cells were incubated with Cell Stimulation Cocktail (eBioscience) for 2 h before adding monensin, and further incubated at 37 °C for a total of 5 h. Cell viability was measured using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific) as per the manufacturer’s instructions. After staining, the cells were acquired and sorted using a FACS Aria III (BD Biosciences) and analysed using FlowJo v10.
Protein quantification analysis: At timepoint 0 h. At the same time, 10⁶ cells/mL FACS-sorted naive CD4 T cell were incubated in vitro using a combination of plate-bound anti-CD3 (2 µg/mL) and soluble anti-CD28 (2 µg/mL) antibodies. As a positive control, CTCs were also cultured in the presence of 10 μM Tunicamycin (Merck) to induce ER stress. After 24 and 48 h, viable cells were isolated by flow cytometry, RNA extracted (Qiagen) and DNA synthesized (Bioline) for subsequent analysis by qPCR. Fold changes in gene expression were compared to those of activated naive CTCs that had not been exposed to Tunicamycin.

Metabolic assessments: Splenic total CD4⁺ T cells were isolated by magnetic sorting using commercial kit (Miltenyi Biotec) according to the manufacturer’s protocol. Cells were plated at a density of 1 × 10⁶ cells/mL and activated with Mouse T- Activating CD3/CD28 beads according to the manufacturer’s manual (Thermo Fisher Scientific) in RPMI (Merck), supplemented with 10% Heat Inactivated FCS (Invitrogen) and 1% Penicillin-Streptomycin (Merck) for 24 h. For all the following analyses, live cell stains were performed in situ followed by incubation in the dark, in a humidiﬁed, gassed (5% CO2) incubator at 37 °C for 30 min. Active mitochondria were measured by adding 0.5 ng/mL Mitotracker DR (Invitrogen), while Mitofil (Enzo Life Sciences) was added to measure total mitochondria, according to the manufacturer’s protocol. Reactive oxygen species was measured by adding 5 μM MitoSox (ThermoFisher Scientiﬁc) to the cell cultures. Lipid droplets were analysed by the addition of 1 μg/mL Nile red, for measurement of palmitate uptake cells were incubated with 1 μg/mL BODIPY-FL-C16 (ThermoFisher Scientiﬁc) and for uptake of glucose 5 μg/6-NBDG (Invitrogen) was added. Apoptotic cells were detected using an Annexin-V/7AAD kit (BioLegend) with or without the addition of 1 μg/L N-Acetyl-L-cysteine (Merck). Mitochondrial membrane potential (Δψm) was measured with 2 μM JC-1 dye (ThermoFisher Scientiﬁc) by flow cytometry according to the manufacturer’s directions. All staining experiments were performed at least 3 times with biological replicates.

Measurement of metabolic ﬂux: Cellular metabolism was measured using an XFp96 cellular ﬂux analyzer system from Seahorse Bioscience. OXPHOS was measured using a Mitostress test kit (Agilent Technologies) according to the manufacturer’s instructions. Primary T cells, 3 × 10⁵ per well, were cultured in RPMI with no sodium bicarbonate and 1% FCS, 20 mM glucose, 2 mM pyruvate and 50 μM β mercaptoethanol (pH 7.4) at 37 °C for these assays. Glycolysis was measured via ECAR measurements from the mitostress test data. Final drug concentrations used in the Seahorse assays: Oligomycin 1 μM, FCCP 1.5 μM, Rotenone and Antimycin A both at 1 μM. Primary data were analysed using Wave desktop software from Agilent Technologies.

Imaging flow cytometry: Metabolic function was assessed by a 2 camera, 12 channel ImageStream X MkII (Ammis Corporation) with the 60x Multimag objective and the extended depth of field option providing a resolution of 0.3 μm per pixel and 12°/mm. The side scatter laser was turned off to allow channel 6 to be used for PE-Cy7. Fluorescent excitation lasers and powers used were 405 nm (50 mW), 488 nm (100 mW) and 643 nm (100 mW). Bright ﬁeld images were captured on channels 1 and 9 (automatic power setting). A minimum of 30,000 images were acquired per sample using INSPIRE 200 software (Ammis Corporation) and analysed by the IDEAS v 6.2 software (Ammis Corporation) using cells stained with single colour reagents, a colour compensation matrix was generated for all 10 ﬂuorescence channels, run with the INSPIRE compensation settings and analysed with the IDEAS compensation wizard. Images were gated for focus (using the ‘RMS feature’ on the INSPIRE software) and cells selected by selecting for singlet cells (DNA intensity/aspect ratio) and live cells at the time of staining, i.e. LIVE/DEAD aqua low-intensity (channel 8) or low-bright-ﬁeld contrast (channel 1).

Infection with Heligmosomoides polygyrus: CTCs were infected with 200 L3 H. polygyrus larvae by oral gavage. Adult parasites were eliminated by 100 mg/kg of pyrantel embonate (PyrantelP) by oral gavage. For secondary infection, drug-treated mice were challenged with another 200 L3 H. polygyrus larvae by oral gavage 10 days later. Faecal egg counts were used to assess the parasite burden and efﬁcacy of the anti-helminthic treatment throughout the experiment and adult worm burdens were determined using standard procedures. IgG1 serum ELISA: ELISA plates were coated with H. polygyrus excretory/secretory product (HES) at 1 μg/mL in PBS O/N at 4 °C, washed 5 times with PBS containing 0.1% Tween (PBS-T) and then blocked using 2% BSA in PBS for 2 h at 37 °C. The plates were then washed a further 5 times in PBS-T. After incubation, the plates were washed 5 times in PBS-T and a further 2 times in distilled water, after which ABTS substrate (KPL, USA) was added and the plates were read at 405 nm after 2.5 h, using PHERAstar FS plate reader (BMG LABTECH, Germany).

Tuft cell staining: Small intestines were isolated from mice shortly after euthanization and flushed with PBS, the gut was then inverted onto a wooden skewer and placed in 10% neutral buffered formalin (NBF, Merck) solution for 4 h. The tissue...
was sliced longitudinally to remove from the skewer and tissue was then rolled up

using GraphPad Prism software (Graph Pad Software Inc.).

expression of Cre. The two experimental groups (i.e. wild-type versus homozygous

age and gender. Control mice were non-Cre lox::lox animals, hence allowing the

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