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The protein tyrosine phosphatase PTPN22 controls forkhead box protein 3 T regulatory cell induction but is dispensable for T helper type 1 cell polarization

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Summary

Protein tyrosine phosphatases (PTPs) regulate T cell receptor (TCR) signalling and thus have a role in T cell differentiation. Here we tested whether the autoimmune predisposing gene *PTPN22* encoding for a PTP that inhibits TCR signalling affects the generation of forkhead box protein 3 (FoxP3)$^+$ T regulatory (T_{reg}) cells and T helper type 1 (Th1) cells. Murine CD4$^+$ T cells isolated from *Ptpn22* knock-out (*Ptpn22*^{KO}) mice cultured in T_{reg} cell polarizing conditions showed increased sensitivity to TCR activation compared to wild-type (WT) cells, and subsequently reduced FoxP3 expression at optimal-to-high levels of activation. However, at lower levels of TCR activation, *Ptpn22*^{KO} CD4$^+$ T cells showed enhanced expression of FoxP3. Similar experiments in humans revealed that at optimal levels of TCR activation PTPN22 knock-down by specific oligonucleotides compromises the differentiation of naive CD4$^+$ T cells into T_{reg} cells. Notably, *in vivo* T_{reg} cell conversion experiments in mice showed delayed kinetic but overall increased frequency and number of T_{reg} cells in the absence of Ptpn*22*. In contrast, the *in vitro* and *in vivo* generation of Th1 cells was comparable between WT and *Ptpn22*^{KO} mice, thus suggesting PTPN22 as a FoxP3-specific regulating factor. Together, these results propose PTPN22 as a key factor in setting the proper threshold for FoxP3$^+$ T_{reg} cell differentiation.

Keywords: FoxP3$^+$ T_{reg} cells, PTPN22, Th1 cells

Introduction

Regulation of immune responses towards self- and foreign-antigens depends critically upon the function of T regulatory (T_{reg}) cells. Forkhead box protein 3 (FoxP3)$^+$ peripherally derived T_{reg} cells (pT_{reg}) are pivotal in protecting from autoimmunity and excessive inflammation, and appear to act complementarily with other T_{reg} cells, such as thymus-derived T_{reg} (tT_{reg}) and T regulatory type 1 (Tr1) cells [1–3]. FoxP3$^+$ pT_{reg} cells are generated *in vivo* primarily from naive CD4$^+$ (FoxP3$^-$) T cell precursors exposed to the antigen under tolerogenic conditions, upon homeostatic expansion or under specific inflammatory conditions such as helminth infection [4–7]. Transforming growth factor (TGF)-β1 secretion is indispensable for the acquisition of the pT_{reg} cell identity *in vivo* together with interleukin (IL)-2, co-stimulation signals and activation through the T cell receptor (TCR) [8–10]. *In vitro,* naive CD4$^+$ T cells develop readily into suppressive FoxP3$^+$-induced T_{reg} cells (iT_{reg}) after culture with anti-CD3/CD28 monoclonal antibodies (mAbs) in the presence of TGF-β1 and IL-2. The degree or length of TCR activation affects FoxP3 induction. For example, very weak or extremely strong TCR signals are less potent as compared to intermediate TCR signals at inducing FoxP3 in the presence of equal amounts of TGF-β1 and IL-2 [11–16].

PTPN22 encodes for the protein tyrosine phosphatase (PTP), lymphoid phosphatase (LYP) in humans, that is expressed by several cells of the lymphoid and myeloid lineages, and its exact role in T cell development, differentiation and function is unknown [17,18]. A genetic association between a single nucleotide polymorphism (SNP) C1858T corresponding to the single amino acid substitution, R620W, and autoimmunity was first described for type 1 diabetes (T1D) and since then for several other autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus) [19–21]. LYP is involved in TCR signalling, playing important negative regulatory role(s) in T cell activation as supported by data in mice deficient for the homologue gene encoding the Pest-enriched phosphatase (PEP),

or mice knock-in (KI), for the equivalent LYP R620W substitution, PEP R619W (i.e. augmented TCR-induced signalling and cellular activation) [22–25]. *Ptpn22* knock-out (*Ptpn22*^{KO}) mice develop lymphoproliferative disease with no signs of autoimmunity and accumulate memory-phenotype T cells with age [22]. Conversely, PEP R619W KI mice recapitulate several of the features described for *Ptpn22*^{KO} mice, and can progress to lupus-like autoimmune disease if placed on a mixed genetic background [24]. *Ptpn22*^{KO} mice show increased numbers of T_{reg} cells in the periphery, suggesting that PEP alters T_{reg} cells alongside effector cells establishing an equilibrium that does not advance beyond a certain point of immune dysregulation [26,27]. Interestingly, *Ptpn22* silencing in non-obese diabetic mice (NOD) by RNA interference also leads to increased T_{reg} cell numbers in the periphery and confers protection from T1D [28]. The mechanisms by which reduced levels of PEP contribute to an increase in FoxP3$^+$ pT_{reg} cells remain ill defined, as the source of increased T_{reg} cells in *Ptpn22*^{KO} mice has been attributed to thymic output [27] or peripheral conversion [26].

Studies with human subjects carrying the predisposing allele of *PTPN22*, C1858T, have provided controversial data. Lower TCR-induced activation, measured by calcium mobilization and IL-2 cytokine production in carriers compared to donors with the wild-type (WT) *PTPN22* allele, was reported initially [19,29,30]. Based on this, it was concluded that *PTPN22* C1858T is a gain-of-function variant. Later, *PTPN22* C1858T was described as a loss-of-function variant, as the expression of LYP in T and B cells from human carriers was lower at steady state [25], a finding that was disproved by two other studies [24,31]. The function of the human variant has been examined in T and B cells isolated from carriers *in vitro*. Several of these reports again appear to be conflicting, and only a small study addressed the role of *PTPN22* in T_{reg} cell development and function in humans. In this study, *PTPN22* C1858T did not alter peripheral T_{reg} cell numbers but reduced the T_{reg} cell suppressive function [32]. Given the fact that most human and murine studies support a role for PTPN22 in TCR signalling, and the importance that TCR signalling has on FoxP3 T_{reg} cell development [33–35], we investigated the role of PTPN22 in T_{reg} cell induction *in vitro* and *in vivo*.

During TCR-mediated activation in the presence of a specific cytokine microenvironment, naive CD4$^+$ T cells may differentiate towards a specific T helper (Th) cell lineage, such as Th1, Th2 or Th17 (reviewed in [36]). The strength of TCR signalling during *in vitro* activation can determine Th1/2 polarization [37]. Therefore, in the present study we also tested whether *Ptpn22* is involved in Th1 cell polarization. We found that at most levels of TCR activation, naive T cells from *Ptpn22*^{KO} mice differentiated to Th1 cells similarly to those from WT mice. In agreement, in a mouse model of viral infection with lymphocytic choriomeningitis virus (LCMV), LCMV-specific CD4$^+$ T cells from *Ptpn22*^{KO} mice differentiated *in vivo* into Th1 cells similarly to those from WT animals. Taken together, in the current study we report that *PTPN22* is central for FoxP3$^+$ T_{reg} cell induction, but is dispensable for Th1 cell polarization.

Materials and methods

Mice

Homozygous *Ptpn22*^{KO} mice were obtained from Dr Souad Rahmouni (University of Liège, Belgium) after Material Transfer Agreement (MTA) with Genentech (San Francisco, CA, USA), and have been previously described [22]. Mice were interbred with WT C57BL/6 and the heterozygous offsprings were intercrossed to obtain *Ptpn22*^{KO} and WT littermates that were used throughout the study. *Ptpn22*^{KO} and Rag-1-deficient mice (Rag1$^{-/-}$) (C57BL/6 background) were housed under specific pathogen-free conditions in compliance with the guidelines of the San Raffaele Institutional Animal Care and Use Committee (IACUC number 479).

Human donors

Peripheral blood was obtained from healthy donors and patients with long-lasting T1D at the San Raffaele Hospital. All donors who were recruited into this study were Italian residents. The study was approved by the local ethics committee (protocol HSR-TIGET004/DRI003). All subjects who agreed to participate in the study signed an informed consent form before any procedure.

Mouse cell purification

CD4$^+$CD25$^-$ T cells were purified from spleens using the CD4$^+$CD25$^+$ T_{reg} isolation kit and cells were further stained with the fluorescent dye eFluor670 (eBioscience, San Jose, CA, USA) to monitor proliferation. In some experiments, the CD4$^+$CD62L$^+$ naive T cell isolation kit was used to obtain naive CD4$^+$ T cells. All beads and kits were from Miltenyi Biotec (San Diego, CA, USA) and used according to the manufacturer's protocols.

Mouse iT_{reg} and Th1 cell differentiation cultures

For all iT_{reg} and Th1 cell generation, plate-bound anti-CD3 mAb (clone 17A2; BD Biosciences, San Jose, CA, USA) was used at different concentrations together with soluble anti-CD28 mAb (clone 37·51; BD Biosciences) at a 2:1 ratio (e.g. 10 μg/ml anti-CD3 and 5 μg/ml anti-CD28). Cultures were performed in 10% fetal calf serum (FCS) complete RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA). CD4$^+$CD25$^-$ or CD4$^+$CD62L$^+$ naive T cells were

plated at 5×10^5 cells/ml and cultured in 96-well plates for 2, 3 or 4 days. For iT_{reg} cell polarization, 5 ng/ml of rhTGF-β1 (R&D Systems, Minneapolis, MN, USA) and 50 U/ml rhIL-2 (Proleukin; Chiron, Emeryville, CA, USA) were added to the cultures. For Th1 cell polarization, $CD4^+CD62L^+$ naive T cells were cultured in the presence of 10 ng/ml recombinant IL-12 and 200 ng/ml anti-IL-4 mAb. For cytokine detection in iT_{reg} or Th1 cells, LAC [leucocyte activation cocktail containing phorbol myristate acetate (PMA)/ionomycin/GolgiPlug; BD Biosciences] was added at the end of a 4-day culture for 3 h prior to staining, according to the manufacturer's instructions.

Adoptive cell transfer assay

Beads-purified peripheral $CD4^+CD25^-$ T cells (Miltenyi Biotec) from *Ptpn22*^{KO} and WT mice were transferred into $Rag1^{-/-}$ recipient mice through tail vein injection. At different time-points after transfer, weight was monitored and blood was withdrawn retro-orbitally. Recipients were sacrificed 45 days after transfer and T cells were analyzed by flow cytometry.

Infection with LCMV and Th1 analysis

Mice were infected with 10^4 plaque-forming units (PFU) LCMV Armstrong (Arm), as described previously [38]. At 8 days post-infection, spleens were harvested and lymphocytes were activated for 3 h with the class II LCMV-specific peptide glycoprotein $(GP)_{61–80}$ (2 μg/ml) in the presence of brefeldin A, as described previously [39].

Human *in vitro* T_{reg} cultures and oligonucleotide treatment

Mononuclear cells were prepared from 20 ml of peripheral blood, as described previously [40]. Naive human $CD4^+CD127^+CD25^-$ T cells were fluorescence activated cell sorted (FACS) and cultured in 96-well plates at 5×10^5 cells/ml in X-vivo 15 medium and 5% pooled AB human serum (Lonza, Basel, Switzerland). Cells were stimulated with plate-bound anti-CD3 (OKT3, 10 μg/ml; Jansen-Cilag, Raritan, NJ, USA) and soluble anti-CD28 (1 μg/ml; BD Biosciences) mAbs in the presence of 5 ng/ml rTGF-β1 and 100 U/ml rIL-2 for iT_{reg} cell induction and analysed 3, 4 and 5 days after culture. As control, cells were activated similarly in the presence of solely rIL-2 or in the absence of any cytokines. PTPN22-specific or control, scrambled, cell-permeable oligonucleotides were purchased from Gene Tools (Philomath, OR, USA) and used as described previously [41]. In some experiments, cells were stained with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) to monitor proliferation, as described previously [40].

Quantitative reverse transcription–polymerase chain reaction (RT–PCR)

Relative expression levels of PTPN22 mRNA were measured in human iT_{reg} cells treated with control and specific antisense oligonucleotides for PTPN22 2 days after *in vitro* culture. Cell lysis, cDNA synthesis and quantitative real-time PCR (qPCR) were performed using the TaqMan® Gene Expression Assay (Applied Biosystems, Carlsbad, CA, USA) and the 7900 HT Fast Real Time PCR System (Life Technologies, Carlsbad, CA, USA). Primers were purchased from Applied Biosystems. PTPN22 mRNA expression levels were normalized to those of the housekeeping gene hypoxantine phsophoribosyltransferase (HRPT). For error analysis, the standard deviation (s.d.) was calculated.

Flow cytometry

Cells were stained with anti-CD4, -CD25, -CD69 and -CD127 mAbs (all from BD Biosciences, Biolegend, San Diego, CA, USA and eBioscience) and then intracellularly with anti-FoxP3 and CTLA-4 mAbs (eBioscience). For interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-2, IL-10 and IL-17 cytokine detection from iT_{reg} cultures, the eBioscience FoxP3 Cytofix/Cytoperm kit was used according to the manufacturer's instructions. To detect cytokines in Th1 cell cultures, the Cytofix/Cytoperm kit from BD Biosciences was used. All samples were acquired on a FACSCanto or LSRII flow cytometer (BD Biosciences) and analysed with FlowJo (Tree Star, Ashland, OR, USA) software.

Statistics

Comparisons between groups were performed using the paired or unpaired two-tailed Student's *t*-test using Prism software (GraphPad, San Diego, CA, USA). For all analyses, a two-tailed *P*-value ≤ 0·05 was considered significant.

Results

Ptpn22 controls *in vitro* $FoxP3^+$ T_{reg} cell induction in mice

The degree of TCR activation is key for $FoxP3^+$ T_{reg} cell induction *in vitro*: strong TCR activation correlates inversely with FoxP3 up-regulation, but low TCR activation is inefficient to promote robust FoxP3 expression in iT_{reg} cell polarizing conditions [1,35]. Indeed, only intermediate doses of anti-CD3/CD28 mAbs support optimal FoxP3 induction [42,43]. Accordingly, $CD4^+CD25^-$ T cells from WT mice converted efficaciously into $FoxP3^+$ T_{reg} cells *in vitro* at optimal doses of anti-CD3/28 mAbs (i.e. 5/2·5 μg/ml) in the presence of TGF-β1 and IL-2 4 days after culture

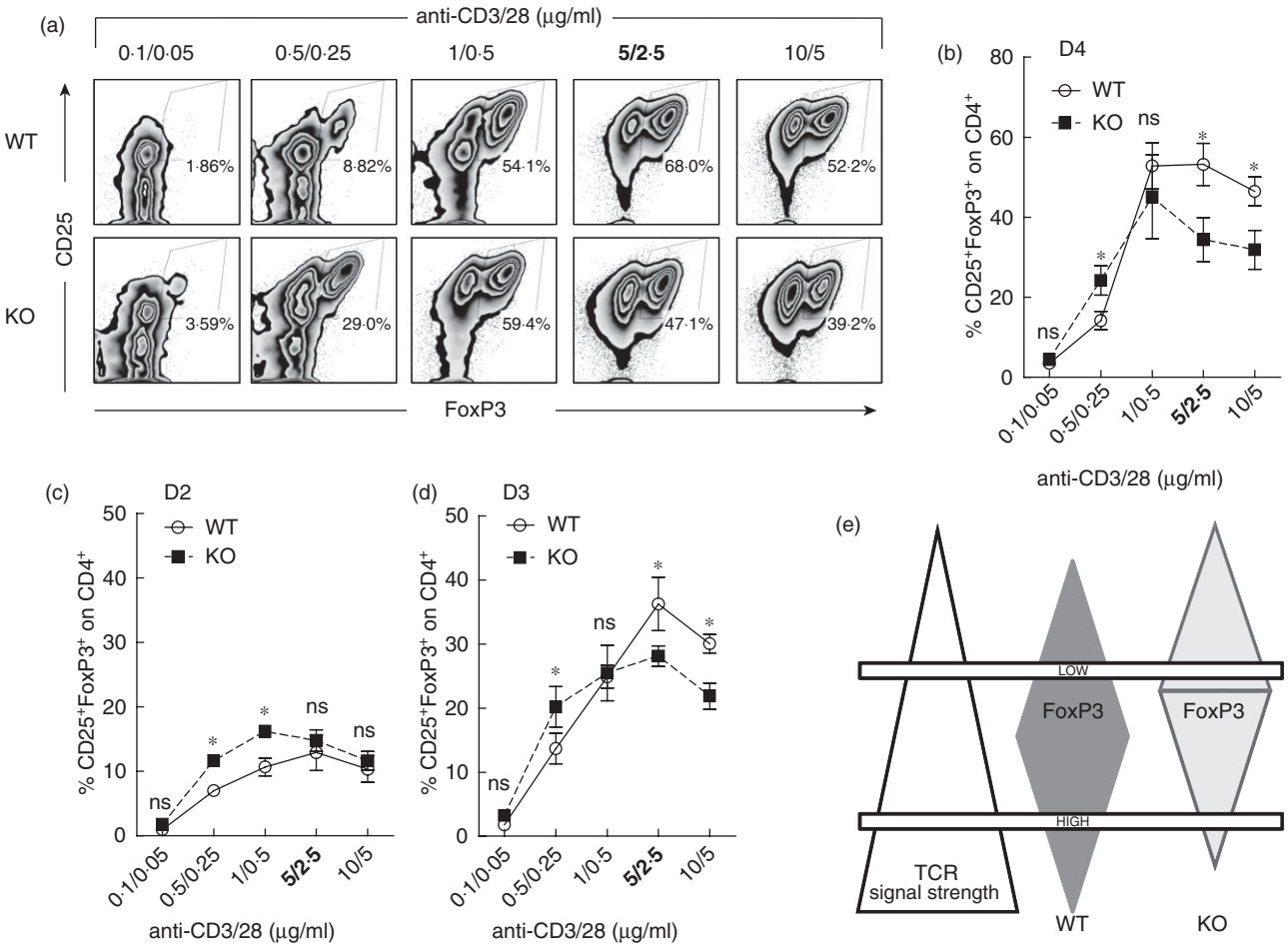

Fig. 1. *Ptpn22* modulates the threshold of T cell activation altering *in vitro* forkhead box protein 3 (FoxP3)$^+$ T regulatory cell induction in mice. $CD4^+CD25^-$ T cells from 8–12-week-old wild-type (WT) or PTPN22 knock-out ($PTPN22^{KO}$) mice were cultured with increasing amounts of anti-CD3/CD28 monoclonal antibodies (mAbs) in the presence of fixed amounts of interleukin (IL)-2 and transforming growth factor (TGF)-β1. (a,b) Expression of CD25 and FoxP3 was evaluated 4 days after culture (gate: $CD4^+$ T cells). In (a), results are representative of five independent experiments with similar results. In (b), cumulative data from all five experiments are shown. (c,d) Frequency of *in vitro*-induced T_{reg} cells ($CD4^+$ $FoxP3^+$) was evaluated 2 days (c) and 3 days (d) after *in vitro* culture. Data from two independent experiments are shown. Dots represent data from individual experiments, whereas bars represent standard error. (e) Proposed model for the effect that *Ptpn22* has on modulating the threshold of T cell receptor (TCR) activation and consequently iT_{reg} cell induction. Pest-enriched phosphatase (PEP) by inhibiting TCR signalling antagonizes FoxP3 at low TCR-mediated activation while it augments FoxP3-inducing signals at higher TCR activating conditions. n.s. = not statistically significant, $^*P < 0{\cdot}05$.

(Fig. 1a). To understand whether PEP contributes to $FoxP3^+$ T_{reg} cell induction *in vitro*, $CD4^+CD25^-$ T cells from $Ptpn22^{KO}$ mice were cultured as those isolated from WT mice. *De-novo* FoxP3 expression was reduced in $CD4^+CD25^-$ T cells lacking *Ptpn22* at optimal and high TCR activation (≥5/25 μg/ml, anti-CD3/28 mAbs). Conversely, as already demonstrated [26], $CD4^+CD25^-$ T cells from $Ptpn22^{KO}$ mice converted more efficiently in $FoxP3^+$ T_{reg} cells at lower doses of TCR activation (Fig. 1a,b).

The kinetic of FoxP3 expression in $Ptpn22^{KO}$ and WT iT_{reg} cell cultures was then assessed. Whereas $CD4^+CD25^-$ T cells from $Ptpn22^{KO}$ mice converted into iT_{reg} earlier and more efficiently when activated with low amounts of anti-CD3/CD28 mAbs (Fig. 1c), lack of *Ptpn22* reduced iT_{reg} cell induction at high levels of activation (Fig. 1d). This data shows that, in the absence of PEP, T cells are more 'ready' to respond to low levels of TCR activation [also confirmed by enhanced up-regulation of CTLA-4 in $Ptpn22^{KO}$-activated T cells (Supporting information, Fig. S1a,b)], and the optimal TCR strength needed to effectively generate $FoxP3^+$ T_{reg} cells is reduced as compared to that needed for inducing iT_{reg} cells in WT mice. Thus, *Ptpn22* sets the threshold for FoxP3 iT_{reg} cell induction in mice by modulating TCR activation (Fig. 1e).

Aged $Ptpn22^{KO}$ mice (>6 months old) develop lymphoproliferative disease and accumulate memory T cells

($CD44^{hi}CD62L^{lo}$) [22]. To define whether memory *Ptpn22*^{KO} T cells, which might contaminate the starting $CD4^+CD25^-$ T cells, inhibit T_{reg} cell induction *in vitro* at optimal FoxP3-inducing culture conditions, the above-mentioned experiments were performed with $CD4^+CD62L^{hi}$ purified naive T cells or with $CD4^+CD25^-$ T cells isolated from younger mice (5–6 weeks old). Similar results were obtained, showing that the reduced FoxP3 expression in cultures with cells from *Ptpn22*^{KO} mice is not due to memory T cell contamination (Supporting information, Fig. S1c). Finally, the resulting $FoxP3^+$ T_{reg} cells were tested for IFN-γ and IL-10 production upon polyclonal stimulation. Similar cytokine production was observed in iT_{reg} cells from *Ptpn22*^{KO} and WT mice, suggesting that the regulation IFN-γ and IL-10 production at increasing TCR activating conditions is not dependent upon the expression of PEP (data not shown).

Ptpn22 does not substantially affect murine Th1 cell polarization *in vitro* and *in vivo*

Ptpn22 may dictate not only the capacity of naive T cells to respond and differentiate into iT_{reg} cells, but also their ability to mature effectively into any other cell lineage. To dissect whether *Ptpn22* is also a key factor for the differentiation of effector T cells, we tested its function in Th1 cell polarization. $CD4^+CD62L^+$ T cells isolated from *Ptpn22*^{KO} and WT mice were cultured under Th1 cell polarizing conditions at various levels of TCR activation. In contrast to $FoxP3^+$ iT_{reg} cell induction, the absence of PEP did not impinge significantly upon the differentiation of Th1 cells, measured by the production of IFN-γ-and TNF-α 4 days after induction. Interestingly, higher Th1 cell differentiation could be achieved when differentiation was induced with the lowest amount of anti-CD3-mediated activation (Fig. 2a,b). This finding underscores that at very low activating TCR conditions, *Ptpn22*^{KO} T cells are more prone to differentiate into Th1 cells. To test whether *Ptpn22* is implicated in Th1 cell differentiation *in vivo*, the LCMV model of acute viral infection was used [44]. Upon infection with LCMV protective innate and adaptive immune responses emerge, of which the $GP_{61–80}$-specific Th1 cell effector response predominates among $CD4^+$ T cells [45]. The absence of *Ptpn22* did not affect the frequency and number of IFN-γ and TNF-α co-producing (Th1) $GP_{61–80}$-specific $CD4^+$ T cells 8 days after infection with LCMV (Fig. 2c,d). Taken together, these results suggest that *Ptpn22* is not involved significantly in Th1 cell lineage commitment *in vitro* and *in vivo*.

PTPN22 is key for *in vitro* $FoxP3^+$ T_{reg} cell induction in humans

TGF-β1 also promotes the differentiation of $CD4^+$ T cells into FoxP3-expressing T cells in humans [46,47]. To investigate whether PTPN22 is key for FoxP3 induction also in human T cells, $CD4^+CD127^+CD25^-$ T cells were isolated from peripheral blood of healthy donors (HD) and cultured *in vitro* under $FoxP3^+$ iT_{reg} cell conditions. To knock-down the expression of PTPN22, PTPN22-anti-sense or control (CTRL) non-targeting oligonucleotides [41] were added to the iT_{reg} cell cultures. Considering that the oligo-treated cells survived similarly in culture irrespective of the oligonucleotides used (data not shown), comparisons between CTRL and PTPN22-specific oligo-treated cultures were made. All HD were screened for the *PTPN22* polymorphism C1858T, and were homozygous for the WT allele, 1858C/C (Supporting information, Table S1). An average of 50% reduction in $CD25^+$ $FoxP3^+$ T cell induction was observed 5 days after culture when PTPN22 mRNA was down-modulated compared to cultures treated with control oligonucleotides (Fig. 3a,b). Additional analysis at an earlier culture time (i.e. 3 and 4 days) confirmed this reduction (Fig. 3c). Control oligonucleotides had no significant effect on $FoxP3^+$ T_{reg} cell induction compared to untreated cells (data not shown). The same data was generated using cells from patients with established T1D (Fig. 4). All patients were negative for the predisposing allele C1858T (Supporting information, Table S2). The ability to up-regulate FoxP3 in untreated $CD4^+CD25^-$ T cells from T1D patients was identical to that in HD (data not shown). Considering that PTPN22-anti-sense oligonucleotides reduced PTPN22 mRNA expression by approximately 50% in these primary T cell cultures (Supporting information, Fig. S2a,b), it is likely that a more efficient PTPN22 reduction might lead to further inability in promoting FoxP3 expression *in vitro*. Overall, these data support that PTPN22 is essential for human $FoxP3^+$ iT_{reg} cell induction *in vitro*.

Ptpn22 determines murine $CD4^+$ T cell expansion and pT_{reg} development *in vivo*

Ptpn22^{KO} mice have increased frequency of FoxP3-expressing T_{reg} cells in secondary lymphoid organs, spleen and lymph nodes ([26,27] and our unpublished observations). Enhanced conversion of naive $CD4^+$ T cells into $FoxP3^+$ pT_{reg} cells could have contributed to their accumulation or an increased rate of thymic production and/or turnover in the periphery. To assess whether *Ptpn22* also affects $FoxP3^+$ pT_{reg} development *in vivo*, we compared the ability of $CD4^+$ T cells from *Ptpn22*^{KO} and WT mice to differentiate into pT_{reg} cells in an *in vivo* model of lymphopenia-induced differentiation [48]. $CD4^+CD25^-$ T cells purified from 8–12-week-old *Ptpn22*^{KO} and WT mice were labelled with eFluor670 and injected intravenously into $Rag1^{-/-}$ mice (Fig. 5a). Cells from *Ptpn22*^{KO} and WT mice proliferated in the peripheral blood to a similar extent in the first 3 and 7 days after transfer (data not shown). As this model of pT_{reg} cell conversion results in mild and transient inflammatory bowel disease (IBD) [48], the weight of

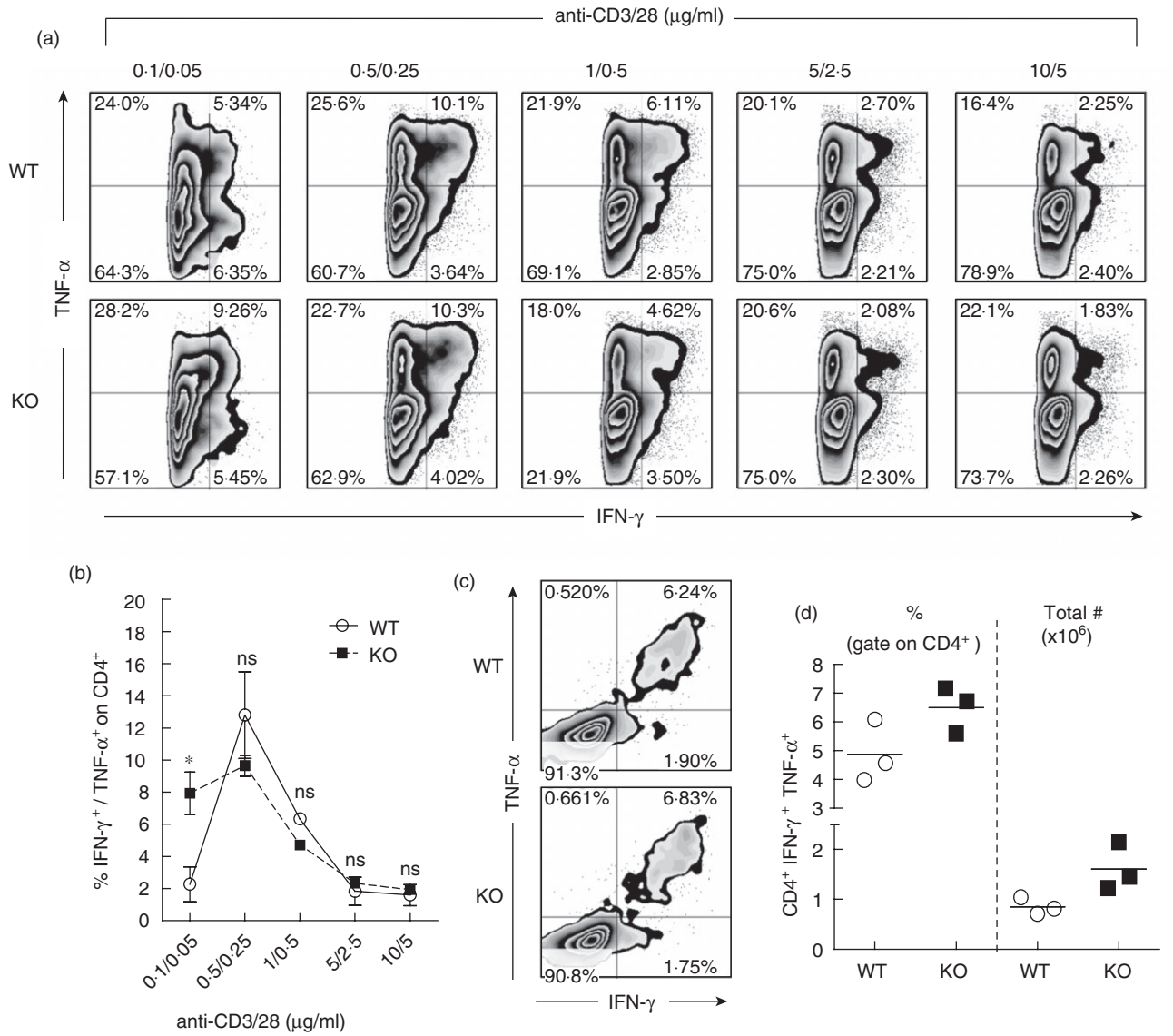

Fig. 2. *Ptpn22* does not significantly alter the T helper type 1 (Th1) cell polarization in mice *in vitro* or *in vivo*. $CD4^+CD62L^+$ T cells from PTPN22 knock-out ($PTPN22^{KO}$) and wild-type (WT) mice were cultured under Th1 cell polarizing conditions. (a) Dot-plots showing representative tumour necrosis factor (TNF)-α and interferon (IFN)-γ production (gate: $CD4^+$ T cells) are shown. (b) Results from two experiments are shown. Dots represent data from individual experiments, whereas bars represent standard error. (c,d) Eight to 10-week-old $Ptpn22^{KO}$ and WT mice were infected with lymphocytic choriomeningitis virus (LCMV) and splenocytes were analysed 8 days later for the production of TNF-α and IFN-γ after stimulation with the class II LCMV-specific epitope $GP_{61–80}$. Representative flow cytometry plots are shown (c). Frequencies (left) and total numbers (right) of Th1 IFN-γ^+ TNF-α^+ $GP_{61–80}$-specific cells are shown (one representative experiment out of three with similar results, each symbol represents individual mice) (d). n.s. = not statistically significant; $^*P < 0{\cdot}05$.

recipient mice was recorded. $Rag1^{-/-}$ mice that received T cells from $Ptpn22^{KO}$ mice showed more weight loss 14 days after transfer (Fig. 5b). In line with this, $CD4^+CD25^-$ T cells from $Ptpn22^{KO}$ mice converted into $FoxP3^+$ pT_{reg} cells less efficiently than those from WT mice 14 days after transfer. However, a trend of increased circulating pT_{reg} cell frequency in mice receiving cells from $Ptpn22^{KO}$ mice was observed 45 days after transfer (Fig. 5c).

The frequencies and total numbers of $CD4^+$ T cells and $FoxP3^+$ pT_{reg} cells were also measured in the spleen (SPL) and mesenteric lymph nodes (MLN) 45 days after cell transfer. pT_{reg} cell frequency and number was higher in the absence of *Ptpn22*, suggesting increased proliferation and/or survival (Fig. 5d–f and Supporting information, Fig. S3a,b). The majority of transferred $CD4^+$ T cells expressed memory-phenotype due probably to homeostatic expansion, but no differences in the proportion of memory cells between $Ptpn22^{KO}$ and WT cell transfers were seen (data not shown).

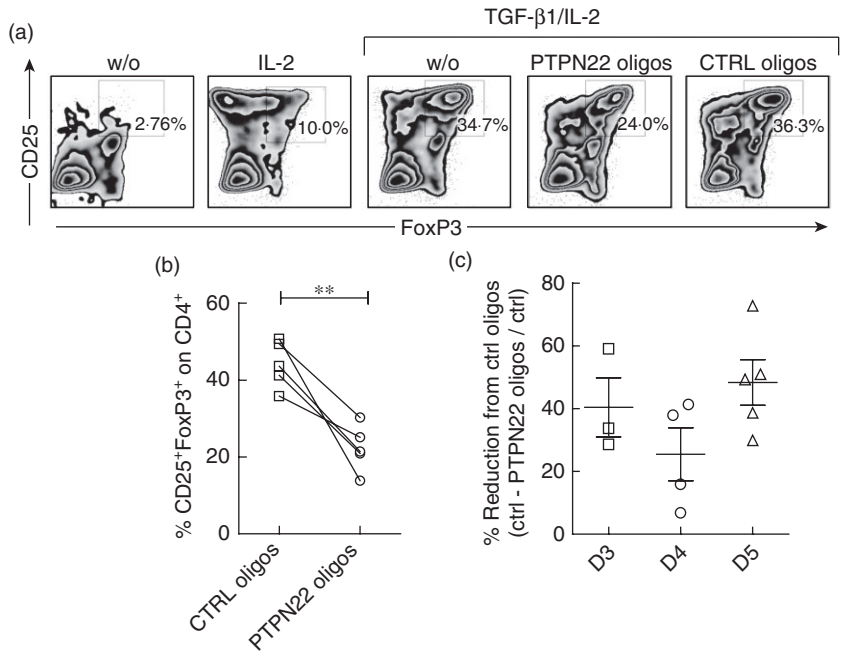

Fig. 3. PTPN22 knock-down inhibits *in vitro* forkhead box protein 3 (FoxP3)$^+$ T regulatory cell induction in human CD4$^+$ T cells from healthy donors. (a) Human CD4$^+$CD127$^+$CD25$^-$ T cells were cultured under *in vitro*-induced (iT$_{reg}$) [transforming growth factor (TGF)-β1/interleukin (IL)-2] and control (w/o, IL-2) conditions. PTPN22-specific and control (CTRL) oligonucleotides were added to the culture. CD25 and intracellular FoxP3 expression evaluated on gated CD4$^+$ T cells after 5 days of culture are shown (one representative of five experiments is presented). (b) Percentages of CD4$^+$CD25$^+$FoxP3$^+$ induction in all five experiments are presented. Lines connect the sets of experiments done in parallel. (c) Percentage reduction of iT$_{reg}$ cell induction in CD4$^+$ T cells treated with PTPN22-specific anti-sense oligonucleotides compared to CTRL oligos at 3, 4 and 5 days after culture [(%CD4$^+$CD25$^+$FoxP3$^+$ T cells CTRL oligos culture-%CD4$^+$CD25$^+$FoxP3$^+$ T cells PTPN22-oligos culture)/%CD4$^+$CD25$^+$FoxP3$^+$ T cells CTRL oligos culture]. Each symbol represents one donor. Bars represent standard deviation. $^{**}P < 0{\cdot}005$, paired *t*-test.

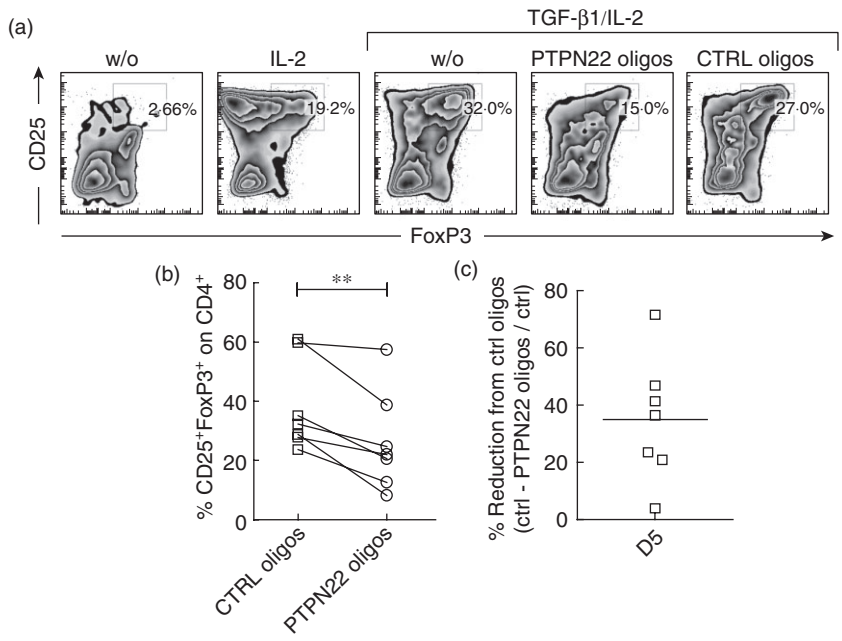

Fig. 4. PTPN22 down-modulation reduces *in vitro* forkhead box protein 3 (FoxP3)$^+$ T regulatory cell induction in CD4$^+$ T cells from patients with type 1 diabetes (T1D). (a) CD4$^+$CD127$^+$CD25$^-$ T cells from patients with established T1D were cultured under *in vitro*-induced (iT$_{reg}$) and control conditions as described in Materials and methods. PTPN22-specific and control (CTRL) oligonucleotides were added to the culture. CD25 and intracellular FoxP3 were evaluated on gated CD4$^+$ T cells after 5 days of culture. One representative experiment out of five is shown. (b) Percentages of CD4$^+$CD25$^+$FoxP3$^+$ induction in all seven donors with T1D are presented. Lines connect the sets of experiments done in parallel. (c) Percentage reduction of iTreg cell induction in CD4$^+$ T cells treated with PTPN22-specific anti-sense oligonucleotides compared to CTRL oligos 5 days after culture [(%CD4$^+$CD25$^+$FoxP3$^+$ T cells CTRL oligos culture-%CD4$^+$CD25$^+$FoxP3$^+$ T cells PTPN22-oligos culture)/%CD4$^+$CD25$^+$FoxP3$^+$ T cells CTRL oligos culture]. Each open square represents one donor. $^{*}P < 0{\cdot}05$.

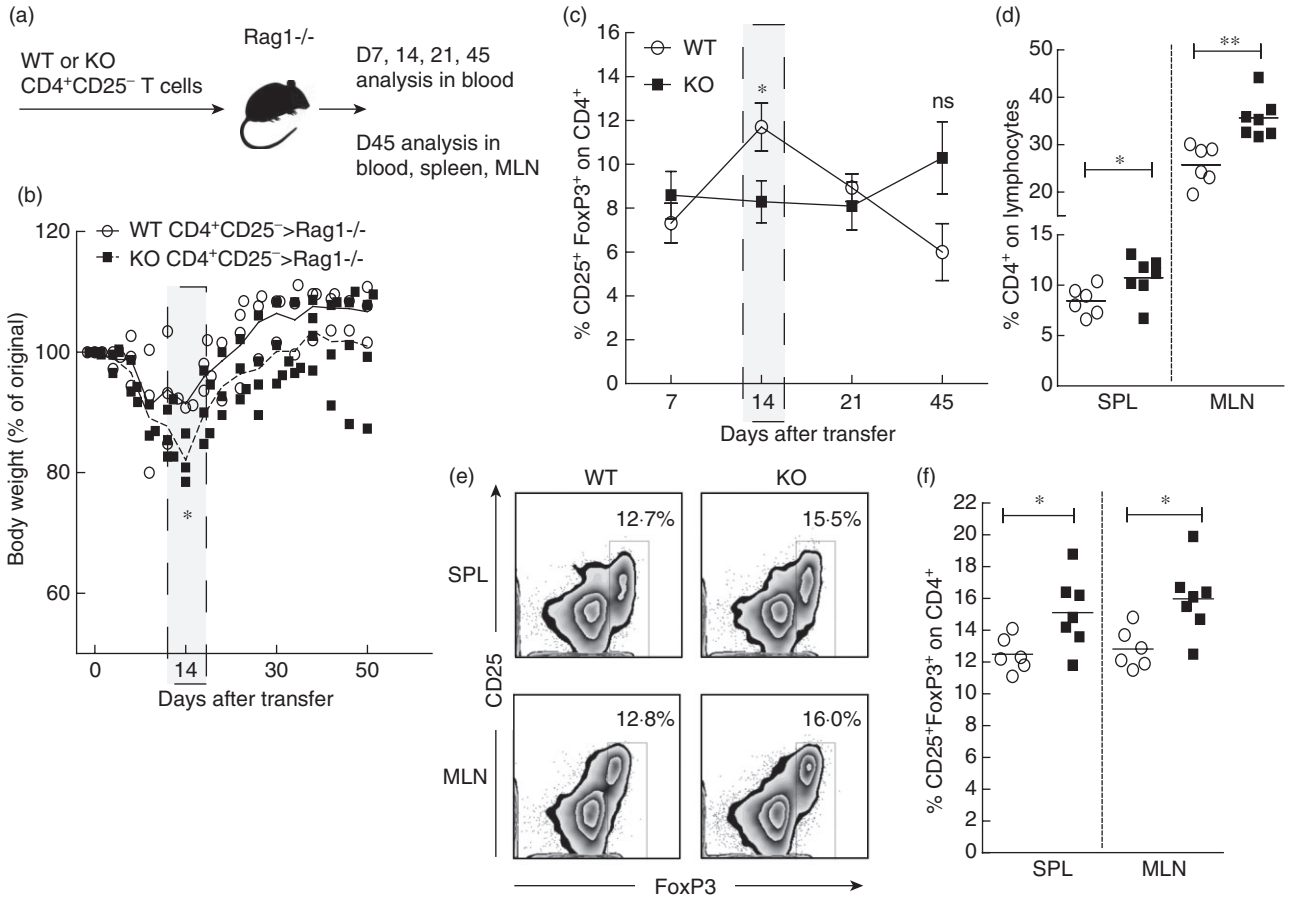

Fig. 5. *Ptpn22* controls CD4+ T cell expansion and accumulation of forkhead box protein 3 (FoxP3)+ peripherally derived T regulatory cells in mice *in vivo*. (a) $CD4^+CD25^-$ purified splenocytes from PTPN22 knock-out ($PTPN22^{KO}$) and wild-type (WT) 8–12-week-old mice were injected intravenously into $Rag1^{-/-}$ recipient mice (1×10^6 cells per mouse total). The frequency of $CD4^+CD25^+$ in transferred cells was comparable before injection (input). The presence of WT and KO cells in the blood of the recipients was monitored on day 1 and was comparable (WT, 0·4936 ± 0·04; KO, 0·5663 ± 0·05). (b) Body weight of $Rag1^{-/-}$ recipient mice given 1×10^6 $CD4^+CD25^-$ T cells from *Ptpn22*^{KO} or from WT mice. Average weight loss per group is shown with a line. (c) At 7, 14, 21 and 45 days after transfer, $CD4^+CD25^-$ cells from *Ptpn22*^{KO} and WT mice were studied with flow cytometry in the peripheral blood of $Rag1^{-/-}$ recipients for *in vivo* conversion into FoxP3-expressing peripherally derived (p)T_{reg} cells. (d) Forty-five days after injection, the spleen (SPL) and mesenteric lymph nodes (MLN) were analysed by flow cytometry for the expansion of $CD4^+$ T cells. Graph shows results from multiple mice. The up-regulation of CD25 and FoxP3 was used to determine the conversion into FoxP3-expressing pT_{regs} in the SPL and MLN of recipient mice 45 days after transfer. Representative plots in (e) illustrate the proportions of $CD25^+FoxP3^+$ peripherally converted T_{reg} cells. Graph in (f) shows results from multiple mice, with open circles representing individual $Rag1^{-/-}$ mice receiving $CD4^+CD25^-$ cells from WT mice and closed squares from *Ptpn22*^{KO} donors. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$.

T_{reg} cells exert their suppressive functions through various mechanisms, some of which include cell-to-cell contact and cytokine secretion [49]. Phenotypical analysis of *Ptpn22*^{KO} and WT pT_{reg} cells showed similar expression of CD25, CD103 and CTLA-4 (data not shown). Furthermore, *Ptpn22*^{KO} and WT $FoxP3^+$ (and $FoxP3^-$) $CD4^+$ T cells produced similar amounts of IFN-γ and IL-10 (Supporting information, Fig. S4). Thus, despite differences in their frequency, *Ptpn22*^{KO} and WT pT_{reg} cells had comparable phenotype and function. In conclusion, 14 days after transfer, $CD4^+$ T cells from *Ptpn22*^{KO} mice convert into $FoxP3^+$ pT_{reg} cells less efficiently than do those from WT mice. Accordingly, recipient mice develop exacerbated IBD. Later, however, pT_{reg} cell conversion peaks in *Ptpn22*^{KO} mice, coinciding with weight gain and disease amelioration.

Discussion

In this study we report that PTPN22 is key for the induction of $FoxP3^+$ T_{reg} cells *in vitro* and *in vivo*, whereas it has no significant effect on Th1 cell differentiation. Our findings indicate that PTPN22 is critical in setting the threshold of TCR activation and, as a consequence, it modulates the levels of FoxP3 induction, while it does not significantly impact Th1 cell polarization. We found that $CD4^+$ T cells from *Ptpn22*^{KO} mice develop into iT_{reg} cell more efficiently at low levels of

TCR activation at three consecutive time-points, in agreement with previous studies [26]. At higher levels of TCR activation, however, the absence of *Ptpn22* reduces FoxP3 induction by overactivating the T cells. Under optimal/strong TCR-activating conditions, human $CD4^+$ T cells treated with anti-sense PTPN22-specific oligonucleotides are also less competent in up-regulating FoxP3. PTPN22 also affects the kinetic of FoxP3 expression *in vivo*; $CD4^+$ T cells from *Ptpn22*^{KO} mice convert less efficiently into pT_{reg} cells initially but expand and accumulate overall at higher numbers compared to WT. In contrast to iT_{reg} cells, *in vitro* Th1 cell polarization is not altered significantly in the absence of *Ptpn22*, except at very low doses of T cell activation.

In vivo pT_{reg} cell formation is mediated by signals derived from the TCR, but also from cytokine receptors [49]. Based on the available reported data [23], the absence of *Ptpn22* probably altered the kinetic of $FoxP3^+$ pT_{reg} cell formation by modulating the TCR-activation levels, rather than affecting other signalling pathways. However, as the levels of TCR activation can also influence the generation of T effector (T_{eff}) cells it is possible that, in the absence of *Ptpn22*, an excessive T_{eff} cell expansion affected pT_{reg} cell formation indirectly, i.e. by outcompeting for space and/or cytokines. Whether pT_{reg} formation in *Ptpn22*^{KO} mice is controlled by such or other mechanism(s) is currently unknown.

$Rag1^{-/-}$ mice receiving $CD4^+$ T cells from *Ptpn22*^{KO} mice transiently develop worse IBD than $CD4^+$ T cells from WT donors at the peak of expansion. Later, however, the conversion rate and/or survival of pT_{reg} cells increases, tipping the balance towards a sustainable T_{eff} to T_{reg} cell ratio. These results suggest that despite the delayed kinetic, pT_{reg} cells from *Ptpn22*^{KO} mice are probably functional. The fact that PEP has a double effect on pT_{reg} cell formation points to a 'yin/yang' role for the phosphatase in T cell tolerance and corroborates the notion that additional defects in other tolerogenic mechanisms are probably required to trigger clinical autoimmunity in PTPN22 R620W human carriers. Despite this, given that protein similarity between mouse and human PTPN22 is less than 80%, PTPN22 might affect iT_{reg} cell formation in humans differently.

It was described recently that PTPN22 is key for driving type I IFN response in myeloid cells. PEP was required for full induction of type I IFNs and, as a consequence, *Ptpn22*^{KO} mice produced decreased numbers of LCMV-specific $CD8^+$ cytotoxic lymphocytes (CTLs) at the peak of the LCMV response [50]. The type I IFN response is essential to many viruses such as LCMV, but seems to affect predominantly the generation of CTLs rather than the $CD4^+$ virus-specific T_{eff} cells [51,52]. We found no differences in the *in vivo* differentiation of LCMV-specific Th1 cells, but we could confirm the reduction in $GP_{33–41}$ CTLs (data not shown). Based on these findings, we can presume that the reduction in $GP_{33–41}$-specific CTLs in this viral infection model is independent of Th1 cells.

Finally, in this study we show that *in vitro* Th1 cell development is not significantly different in the absence of *Ptpn22*, except at very low levels of anti-CD3/28 mAb activation. Interestingly, at low-to-intermediate levels of TCR activation, the absence of Ptpn22 favours FoxP3 T_{reg} cell induction. These findings suggest that, in certain situations, *Ptpn22*^{KO} mice might result as more tolerant, depending on the $Th1/T_{reg}$ cell ratio. Interestingly, *Ptpn22*^{KO} mice were shown to be resistant to experimental autoimmune encephalomyelitis (EAE) [27], but susceptible to dextran sulphate sodium (DSS)-induced colitis [50,53]. While both diseases are mediated mainly by Th17 cells, it is unclear how the lack of *Ptpn22* protected from EAE but exacerbated DSS colitis. In this study we also showed that cytokines, and particularly IL-10, is produced at similar levels by *Ptpn22*^{KO} and WT iT_{reg} cells. These results indicate that *Ptpn22*^{KO} iT_{reg} cells might possess similar suppression properties to WT iT_{reg} cells.

In summary, our results demonstrate that PTPN22 is key for inducible T_{reg} cell generation and that it acts mainly through modulating the threshold of T cell activation. Lack of *Ptpn22* alters Th1 cell differentiation only at low levels of activation, providing a possible explanation for the expanding natural memory T cell pool that characterizes *Ptpn22*^{KO} mice [22].

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Disclosures

The authors have no financial conflicts of interest.

Author contributions

G. F. designed, performed experiments, analysed the data and wrote the paper. T. J., I. D. and S. S. performed some of the experiments. A. L. collected human peripheral blood. N. B. provided human PTPN22-specific oligonucleotides, assisted with the interpretation of the results and corrected the paper. M. B. supervised the study and wrote the paper.

All authors read the manuscript and contributed to the discussion of the findings.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. PTPN22 affects forkhead box protein 3 (FoxP3) induction by inhibiting T cell activation in mice. $CD4^+CD25^-$ T cells from 8–12-week-old wild-type (WT) or PTPN22 knock-out ($PTPN22^{KO}$) mice were cultured under *in vitro*-induced regulatory T cell (iT_{reg}) polarizing conditions with increasing amounts of anti-CD3/CD28 monoclonal antibodies (mAbs). (a) Histogram overlay depicting the levels of CTLA-4 in WT and $PTPN22^{KO}$ $CD4^+$ T cells 4 days after culture. Blue line indicates WT cells, whereas red indicates KO. (b) The mean fluorescence intensity (MFI) levels of cytotoxic T lymphocyte antigen 4 (CTLA-4) in WT and KO cells depicted in (c) shown in a graph. Results from one representative experiment are shown. (c) Cumulative data showing the % difference in $CD25^+FoxP3^+$ induction between WT and KO cells from 10 independent experiments are displayed in a graph. Each coloured line indicates results from independent experiments. In some experiments, naive T cells ($CD4^+CD62L^{hi}$) or cells from young animals (>6 weeks old) were cultured (indicated in the figure, including the experiment that was selected as representative in Fig. 1). Not all anti-CD3/28 activating conditions were tested in each experiment.

Fig. S2. Anti-sense PTPN22-specific oligonucleotide treatment reduces by 50% the endogenous PTPN22 expression levels in human *in vitro*-induced regulatory T cell (iT_{reg}) cultures. (a) Human $CD4^+CD127^+CD25^-$ T cells from healthy donors (HD) were fluorescence activated cell sorted (FACS) and cultured under iT_{reg} polarizing conditions as described in Materials and methods. PTPN22-specific and control (CTRL) oligonucleotides were added at the beginning of iT_{reg} cultures with $CD4^+$ T cells derived from HD. Expression levels of PTPN22 were evaluated 2 days later by real-time quantitative polymerase chain reaction (qPCR), $P < 0{\cdot}05$. (b) Percentage reduction of PTPN22 expression levels in human $CD4^+$ T cells compared to CTRL oligos. Each open square represents individual donor.

Fig. S3. $CD4^+$ T cell expansion and regulatory T cell (T_{reg}) cell development upon lymphopenic expansion in the absence of PTPN22. (a,b) Forty-five days after transfer into lymphopenic $B6.Rag1^{-/-}$ hosts, $CD4^+CD25^-$ T cells from PTPN22 WT and PTPN22 knock-out ($PTPN22^{KO}$) mice were studied for expansion (total number of $CD4^+$ T cells) and peripheral (p)T_{reg} conversion in the spleen (SPL) and mesenteric lymph nodes (MLN). Each symbol represents individual animal and horizontal bar shows the mean of values. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$.

Fig. S4. Cytokine production by peripherally induced regulatory T cells (T_{reg}) and effector T cells (T_{eff}) in the absence of PTPN22. (a,b) Forty-five days after transfer into lymphopenic $B6.Rag1^{-/-}$ hosts, $CD4^+CD25^-$ cells from PTPN22.WT and PTPN22 knock-out ($PTPN22^{KO}$) mice converted into forkhead box protein 3 (FoxP3)-expressing peripherally derived (p)T_{regs} and memory cells in the spleen (SPL) and mesenteric lymph nodes (MLN). Total lymphocytes were activated with leucocyte activation cocktail (LAC) prior to staining intra-cytoplasmatically for FoxP3 and interferon (IFN)-γ (a,b) or interleukin (IL)-10 (c,d)

anti-cytokine antibodies. One representative experiment of two with similar results is shown in (a) and (c) after gating on total $CD4^+$ T cells. Each dot represents individual mice in (b) and (d) and horizontal bar shows the mean of values.

Table S1. Information on healthy donors (HD) used for this study.

Table S2. Information on type 1 diabetic subjects used for study.