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Article

The Phosphatase PTP-PEST Promotes Secondary T Cell Responses by Dephosphorylating the Protein Tyrosine Kinase Pyk2

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SUMMARY

PTP-PEST (encoded by *Ptpn12*) is an intracellular protein tyrosine phosphatase belonging to the same family as LYP. LYP inhibits secondary T cell responses by suppressing Src family protein tyrosine kinases and is implicated in human autoimmunity. To determine the function of PTP-PEST in T cells, we generated mice with a conditionally deleted allele of *Ptpn12*. By removing PTP-PEST in T cells, we determined that PTP-PEST was not necessary for T cell development or primary responses. However, PTP-PEST was required for secondary T cell responses, anergy prevention, and autoimmunity induction. PTP-PEST specifically regulated the phosphorylation of Pyk2, a substrate of the Src family kinase Fyn. It also promoted the formation of T cell homoaggregates, which are known to enhance T cell activation. Thus, PTP-PEST controls Pyk2 activity and is a positive regulator of secondary T cell activation. These data illustrate the critical role of protein tyrosine phosphatases in T cell regulation.

INTRODUCTION

The proline-, glutamic acid-, serine-, and threonine-rich (PEST) family of intracellular protein tyrosine phosphatases (PTPs) includes PTP-PEST (PTPN12), lymphoid tyrosine phosphatase (LYP) (also known as proline-enriched phosphatase [PEP] or PTPN22; hereafter named LYP) and PTP-hematopoietic stem cell fraction (HSCF) (Veillette et al., 2002; Veillette et al., 2009; Bottini et al., 2006). These enzymes are composed of an amino-terminal PTP domain, a central protein-protein interaction domain, and a conserved carboxyl-terminal tail. LYP and PTP-HSCF are expressed in hematopoietic cells, whereas PTP-PEST is expressed in all cell types, albeit in the highest amounts in immune cells.

In immune cells, the best-characterized PEST family member is LYP. LYP is a negative regulator of T cell activation (Cloutier and Veillette, 1999; Cloutier and Veillette, 1996; Hasegawa et al., 2004). This activity correlates with the ability of LYP to dephosphorylate and inactivate Src family protein tyrosine kinases (PTKs) (Lck and Fyn) and ZAP-70, which initiate T cell activation. T cells from LYP-deficient mice are hyperresponsive to T cell receptor (TCR) stimulation (Zikherman et al., 2009; Hasegawa et al., 2004). Intriguingly, this effect is seen in previously activated T cells, but not in naive T cells (Hasegawa et al., 2004). LYP-deficient mice also exhibit an accumulation of effector-memory T cells, an expansion of germinal centers and an increase in antibody amounts. Thus, LYP inhibits T cell activation only in effector-memory T cells. A point mutation in human LYP that affects the ability to regulate TCR signaling is a risk factor for autoimmune diseases like type 1 diabetes, lupus, and rheumatoid arthritis (Bottini et al., 2006; Bottini et al., 2004).

The roles of the other PEST family members in immune cells are not well understood. In nonimmune cells, PTP-PEST can dephosphorylate the cytoskeleton-associated adaptors Cas, PSTPIP-1, and paxillin and the focal adhesion-associated PTKs FAK and Pyk2 (Badour et al., 2004; Davidson and Veillette, 2001; Garton and Tonks, 1999; Coté et al., 1998; Shen et al., 1998; Garton et al., 1996; Cong et al., 2000). It can also regulate cell adhesion, spreading, and migration. Overexpression analyses in lymphoid cells suggested that PTP-PEST may also be an inhibitor of lymphocyte activation (Badour et al., 2004; Davidson and Veillette, 2001; Arimura et al., 2008). This activity was linked to the ability of PTP-PEST to dephosphorylate Src kinases, FAK, Pyk2, Shc, or WASP. Thus, like LYP (Zikherman et al., 2009; Hasegawa et al., 2004), PTP-PEST may be a negative regulator of T cell activation. However, the physiological relevance of this effect has not been confirmed because conventional PTP-PEST-deficient mice exhibit an early embryonic lethality (Sirois et al., 2006).

To address the physiological role of PTP-PEST in T cells, we generated a mouse having a conditionally targeted allele of *Ptpn12*, the gene encoding PTP-PEST. By crossing this mouse with mice expressing the Cre recombinase in T cells, we found that PTP-PEST was not necessary for T cell development and primary T cell responses. However, it was required for secondary T cell activation, inhibition of anergy, and induction of autoimmunity. These activities correlated with the selective capacity of PTP-PEST to dephosphorylate Pyk2, a substrate of Fyn, and to enhance the formation of homoaggregates between T cells.

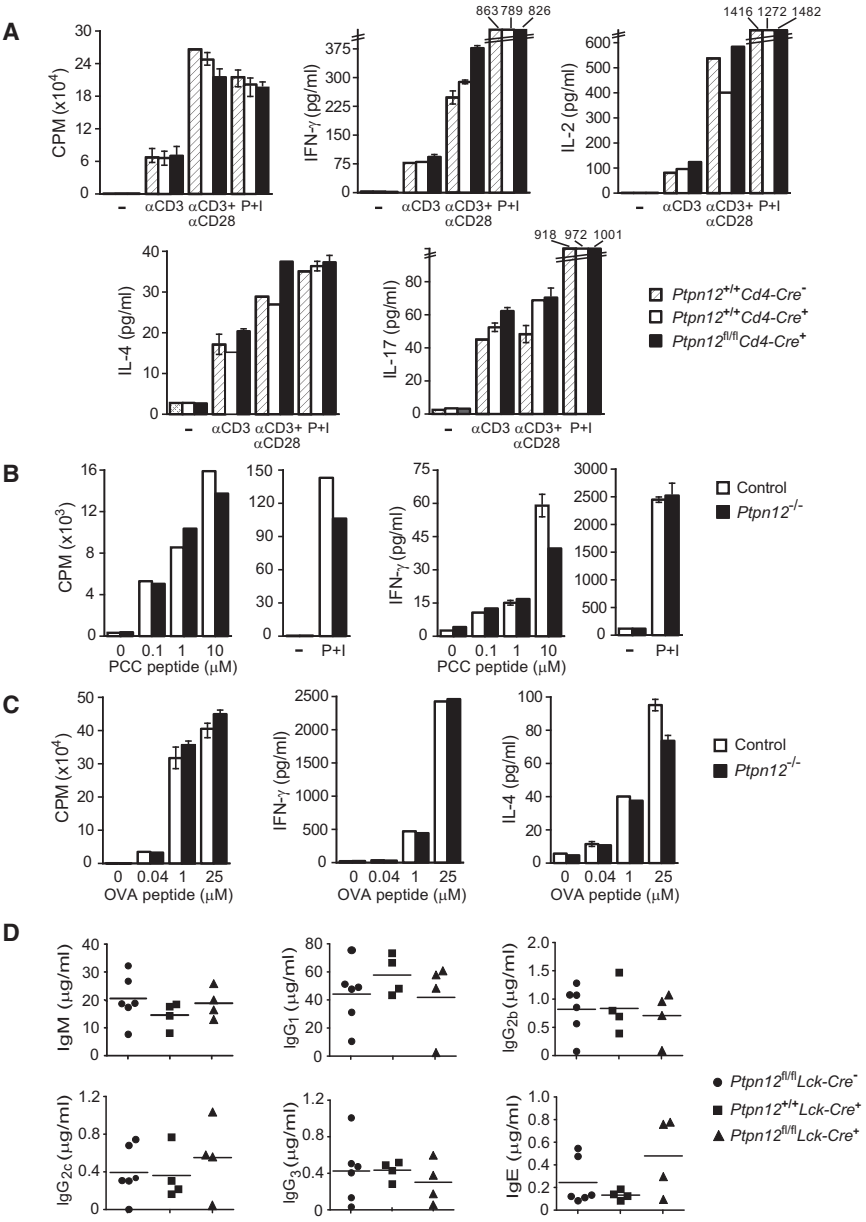


Figure 1. Primary T Cell Responses in PTP-PEST-Deficient Mice

(A) Purified CD4$^+$ T cells were activated in vitro with the indicated stimuli. After 48 hr, proliferation and cytokine production were determined by measuring thymidine incorporation and by ELISA, respectively. Standard deviations of triplicate values are shown. CPM, counts per min; αCD3, anti-CD3; αCD28, anti-CD28; P+I, PMA plus ionomycin. A representative of seven experiments is shown.

(B and C) Purified CD4$^+$ T cells from the indicated mice, expressing the (B) pidgeon cytochrome C (PCC)-specific transgenic TCR AND or (C) the ovalbumin (OVA)-specific transgenic TCR OT-II, were activated with antigenic peptides and splenocytes, or with P+I. Activation was monitored as detailed in (A). Standard deviations of triplicate values are shown. A representative of five experiments (B) or one experiment (C) is shown.

(D) Mice (n = 4–6 in each group) were immunized with trinitrophenyl (TNP)-chicken gamma globulin (CGG) plus alum. After 14 days, TNP-specific serum immunoglobulins (Ig) were measured by ELISA. Symbols represent individual mice. Average values are shown as horizontal bars. Similar results were observed when antibody levels were assessed 7 or 21 days after immunization (data not shown). In all cases, p values were nonsignificant (>0.05). A representative of two experiments encompassing ten conditional PTP-PEST-deficient mice is shown. See also Figure S1 and Table S1.

RESULTS

PTP-PEST Is Not Necessary for Primary CD4$^+$ T Cell Responses

We engineered a mouse with a conditional allele of the gene encoding PTP-PEST (*Ptpn12*) (see Supplemental Experimental Procedures and Figure S1 available online). To determine the role of PTP-PEST in T cells, we bred *Ptpn12*$^{fl/fl}$ mice with mice expressing the Cre recombinase under the control of the *Lck* or *Cd4* promoter. Mice expressing Cre alone or carrying the floxed allele alone were used as "wild-type" controls. Unless specified, the experiments shown used *Ptpn12*$^{+/+}$*Cre*$^+$ mice as controls. Cre-mediated deletion of the floxed alleles resulted in a >95% reduction of the abundance of PTP-PEST in T cells (Figure S1D). No truncated protein was observed (data not shown). PTP-PEST deficiency had no effect on T cell numbers or subpopulations, the numbers or proportions of naive, effector-memory, and CD4$^+$Foxp3$^+$ regulatory T (Treg) cells, and the proportions of natural killer (NK)-T cells (Table S1; data not shown).

To examine the impact of PTP-PEST on T cell activation, we focused our studies on CD4$^+$ T cells. Freshly purified control and PTP-PEST-deficient (hereafter named *Ptpn12*$^{-/-}$) CD4$^+$ T cells were activated in vitro with anti-CD3, without or with anti-CD28. PTP-PEST deficiency had no effect or only a small stimulatory effect (<1.5-fold) on thymidine incorporation and production of interferon (IFN)-γ, interleukin (IL)-2, IL-4 and IL-17 (Figure 1A). PTP-PEST-deficient mice were also crossed with class II major histocompatibility complex (MHC)-restricted TCR transgenic mice AND and OT-II (Kaye et al., 1989; Barnden et al., 1998). Compared to CD4$^+$ T cells from control TCR transgenic mice, *Ptpn12*$^{-/-}$ T cells showed little or no alteration in thymidine incorporation and cytokine production in response to antigenic peptides plus splenocytes (Figures 1B and 1C).

We also examined primary CD4$^+$ T cell responses in vivo by immunizing mice with trinitrophenyl (TNP)-chicken gamma globulin (CGG) and alum and measuring antibodies against the

hapten TNP (Figure 1D). Production of TNP antibodies is dependent on proper $CD4^+$ T cell helper functions. Lack of PTP-PEST in T cells had no effect on the ability to produce TNP antibodies. Similar results were obtained when mice were immunized with ovalbumin (OVA) or a myelin oligodendrocyte glycoprotein (MOG) peptide (see below). Thus, PTP-PEST deficiency had little or no effect on T cell development and differentiation and on primary $CD4^+$ T cell responses.

PTP-PEST Is a Positive Regulator of Secondary $CD4^+$ T Cell Activation

The impact of PTP-PEST deficiency on secondary $CD4^+$ T cell responses was evaluated (Figure 2). $CD4^+$ TCR AND T cells were first activated with anti-CD3 plus anti-CD28 and subsequently restimulated in vitro with antigen and antigen-presenting cells (APCs) (Figure 2A). In comparison to control T cells, previously activated *Ptpn12*$^{-/-}$ T cells exhibited a decrease in thymidine incorporation (to ~50%) and cytokine production (to ~10%–20%) upon restimulation. The latter was true for IFN-γ, IL-2, and IL-4. This was surprising, given that earlier overexpression studies had suggested that PTP-PEST is a negative regulator of T cell activation (Badour et al., 2004; Davidson and Veillette, 2001; Arimura et al., 2008). No effect on production of the immunosuppressive cytokine IL-10 and transforming growth factor (TGF)-β was seen (Figure S2A). Similar results were obtained when the reverse stimulation protocol was used, in which $CD4^+$ TCR AND T cells were first activated with antigen and APCs, and then restimulated with anti-TCR plus anti-CD28 or anti-CD3 plus anti-CD28 (Figure S2B; data not shown). A decreased rate of cell division was also noted when cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (Figure S2C). Likewise, a delay in cell cycle progression was observed when permeabilized cells were stained with propidium iodide (PI) (Figure S2D). These various defects were not due to a loss of transgenic TCR expression (data not shown) nor to an increase in activation-induced cell death (Figure S2E). Furthermore, they did not reflect a global defect in responsiveness, as cells responded normally to phorbol myristate acetate (PMA) plus ionomycin (P+I).

Next, we tested secondary T cell responses by using antigen-specific T cells generated in vivo (Figure 2B). Mice (devoid of transgenic TCR) were injected with OVA and adjuvant. After 9 days, draining lymph node $CD4^+$ T cells were restimulated in vitro with OVA and splenocytes. *Ptpn12*$^{-/-}$ T cells demonstrated a marked decrease (to ~10%–20%) in thymidine incorporation, as well as in secretion of IFN-γ and IL-2. These defects did not relate to a lack of primary $CD4^+$ T cell response in vivo, as judged by staining of lymph node cells with class II MHC tetramers loaded with an OVA peptide or by short-term restimulation with P+I, followed by intracellular staining for IFN-γ (Figure 2C; Figure S2F). Effector-memory T cells, but not naive T cells, undergo rapid production of IFN-γ after short-term restimulation with P+I (Hatton et al., 2006). Moreover, there was no defect in the appearance of OVA antibodies (Figure S2G).

To explain these findings, we examined whether PTP-PEST deficiency might be enhancing the number or activity of Treg cells (Figure 2D). There was no greater number of $CD4^+Foxp3^+$ Treg cells in lymph nodes of OVA-immunized PTP-PEST-deficient mice, compared to OVA-immunized control mice. Moreover, in in vitro suppression assays, $CD4^+$ T cells from OVA-immunized PTP-PEST-deficient mice failed to suppress thymidine incorporation and cytokine production of $CD4^+$ T cells from OVA-immunized control mice (Figure 2E). In fact, the defect in thymidine incorporation of *Ptpn12*$^{-/-}$ T cells appeared to be corrected when they were added to cells from control mice. This rescue could be caused by the ability of control T cells to secrete growth-stimulating cytokines such as IL-2 or to promote contacts between T cells (see below). Therefore, PTP-PEST was intrinsically required for the ability of T cells to respond to secondary stimulation.

Selective Deregulation of Pyk2 and Rac1 in *Ptpn12*$^{-/-}$ T Cells

To understand the mechanism by which PTP-PEST enhanced secondary T cell responses, we examined the effect of PTP-PEST deficiency on TCR-triggered signals (Figure 3). T cells were stimulated or not with anti-CD3 alone or plus anti-CD28, and protein tyrosine phosphorylation was monitored (Figure 3A). PTP-PEST deficiency had no effect on tyrosine phosphorylation of most substrates in resting or TCR-stimulated T cells. The only exception was a ~115 kilodalton (kDa) protein, which exhibited enhanced tyrosine phosphorylation in *Ptpn12*$^{-/-}$ T cells. This was seen in fresh ex vivo and previously activated T cells, as well as in resting and, to a lesser extent, TCR-stimulated T cells. Immunoblotting with phospho-Pyk2-specific antibodies suggested that p115 was Pyk2, a member of the FAK family of PTKs that is highly expressed in hematopoietic cells and undergoes tyrosine phosphorylation by Fyn upon TCR stimulation (Ostergaard et al., 1998; Qian et al., 1997). This notion was confirmed both by immunoprecipitation and by depletion experiments using anti-Pyk2 (Figure 3B; Figure S3A). Pyk2 was likely a direct substrate of PTP-PEST, given that recombinant PTP-PEST was apt at dephosphorylating Pyk2 in vitro (Figure S3B). PTP-PEST deficiency had little or no effect on tyrosine phosphorylation of other known PTP-PEST substrates such as FAK, CasL, Cas, PSTPIP-1 and Shc (Figure 3B). It also did not interfere with TCR-triggered activation of the kinases Erk, JNK, p38, and Akt and protein kinase C (PKC)-θ and IκBα activation and degradation (Figure 3C). Similar results were obtained with $CD4^+$ TCR AND T cells previously activated with antigen-MHC (Figure S3C). Likewise, lack of PTP-PEST had no effect on capping of the TCR complex or actin polymerization (Figures S3D and S3E). Nonetheless, it resulted in a modest, albeit reproducible, decrease in Ca^{2+} mobilization (Figure 3D). More noticeably, it led to an increase in basal activity of the small guanine nucleotide-binding protein Rac1 (a known effector of Pyk2 [Gismondi et al., 2003]) and, paradoxically, a decrease in TCR-induced activation of Rac1 (Figure 3E). The impact of PTP-PEST deficiency on baseline Pyk2 tyrosine phosphorylation was not corrected by P+I (Figure S3F), arguing that P+I was rescuing functional responses (Figure 2) by acting downstream of Pyk2. Hence, PTP-PEST deficiency caused a selective increase in Pyk2 tyrosine phosphorylation, which was accompanied by deregulation of Rac1.

Compromised Homoaggregate Formation by *Ptpn12*$^{-/-}$ T Cells

PTP-PEST, like Pyk2 and Rac1, regulates cell adhesion, at least in nonlymphoid cells (Veillette et al., 2009; Larsen et al., 2003;

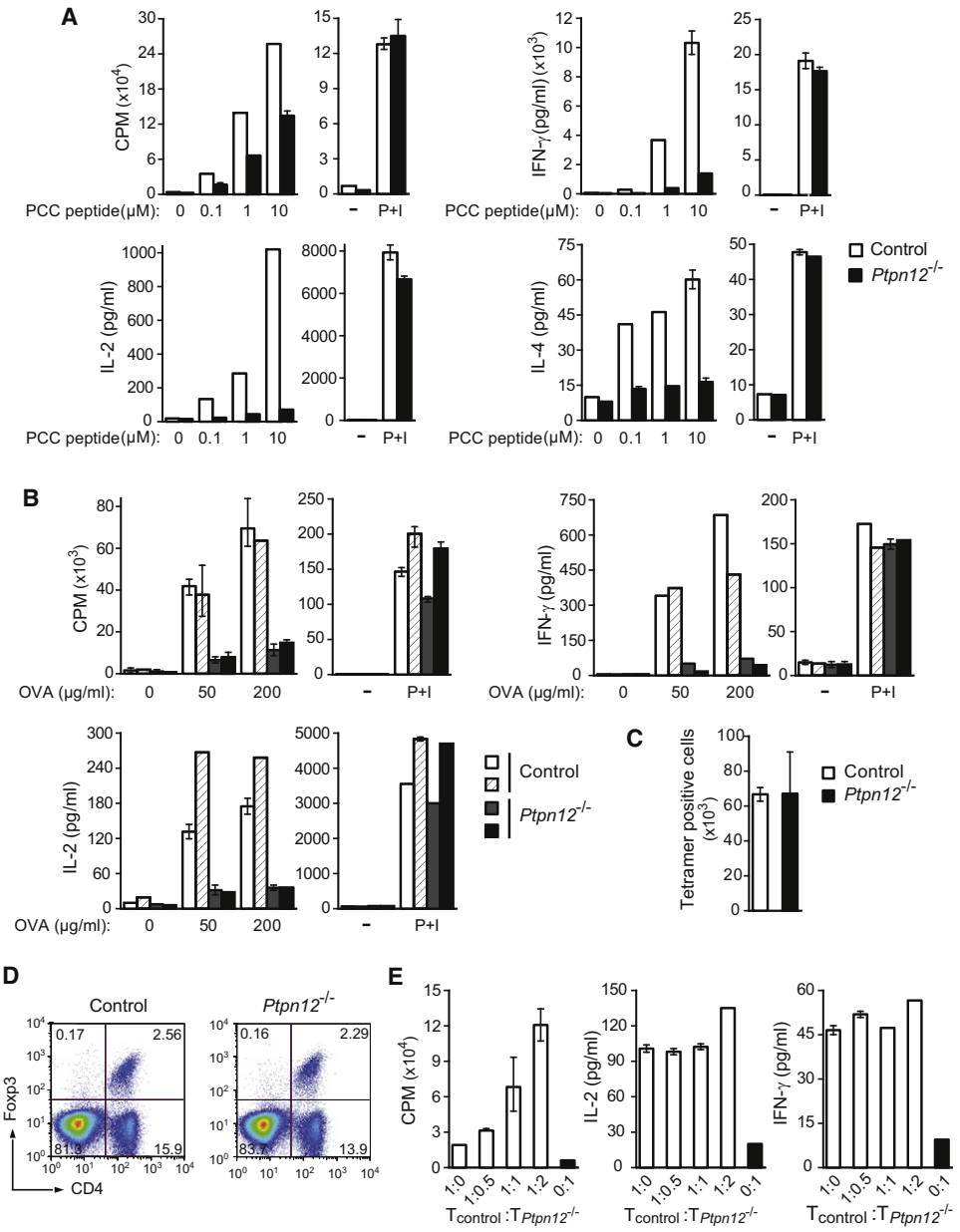


Figure 2. Severely Compromised Secondary T Cell Responses in PTP-PEST-Deficient Mice

(A) Purified CD4$^+$ T cells from TCR AND transgenic mice were first activated in vitro with anti-CD3 plus anti-CD28. They were then restimulated with PCC peptide and irradiated splenocytes, or with PMA plus ionomycin (P+I). Thymidine incorporation and cytokine production were monitored. Standard deviations of triplicate values are shown. A representative of eight experiments is shown.

(B–D) Mice (two representative mice from each group) were immunized with OVA and complete Freund adjuvant in the foot pad. As shown in (B), after 9 days, CD4$^+$ T cells were isolated from popliteal lymph nodes and restimulated with OVA and splenocytes, or P+I. After 4–5 days (for OVA restimulation) or 2 days (for P+I stimulation), activation was monitored as detailed in (A). Standard deviations of triplicate values are shown. As shown in (C), CD4$^+$ T cells from draining lymph nodes of immunized mice were stained with class II MHC-tetramers loaded with $OVA_{323-339}$ peptide and analyzed by flow cytometry. As the control, cells were stained with tetramers loaded with an irrelevant peptide. The total numbers of tetramer-positive cells in draining lymph nodes are shown. As shown in (D), the relative abundance of CD4$^+$Foxp3$^+$ T cells in draining lymph nodes was assessed by flow cytometry. A representative of eight (B), three (C), and two (D) experiments is shown.

(E) Suppressor activity in draining lymph nodes was assessed by mixing CD4$^+$ T cells from control mice with CD4$^+$ T cells from PTP-PEST-deficient mice (at the indicated ratios), prior to in vitro restimulation with OVA, as described in (B). A representative of two experiments is shown. See also Figure S2.

Bishop and Hall, 2000; Avraham et al., 2000). Therefore, we tested whether PTP-PEST might be affecting the ability of T cells to contact APCs (Figure S4A). Lack of PTP-PEST had no influence on the ability of previously activated CD4$^+$ T cells to form conjugates with B cells. This was seen over a range of antigen concentrations and at several times after antigen

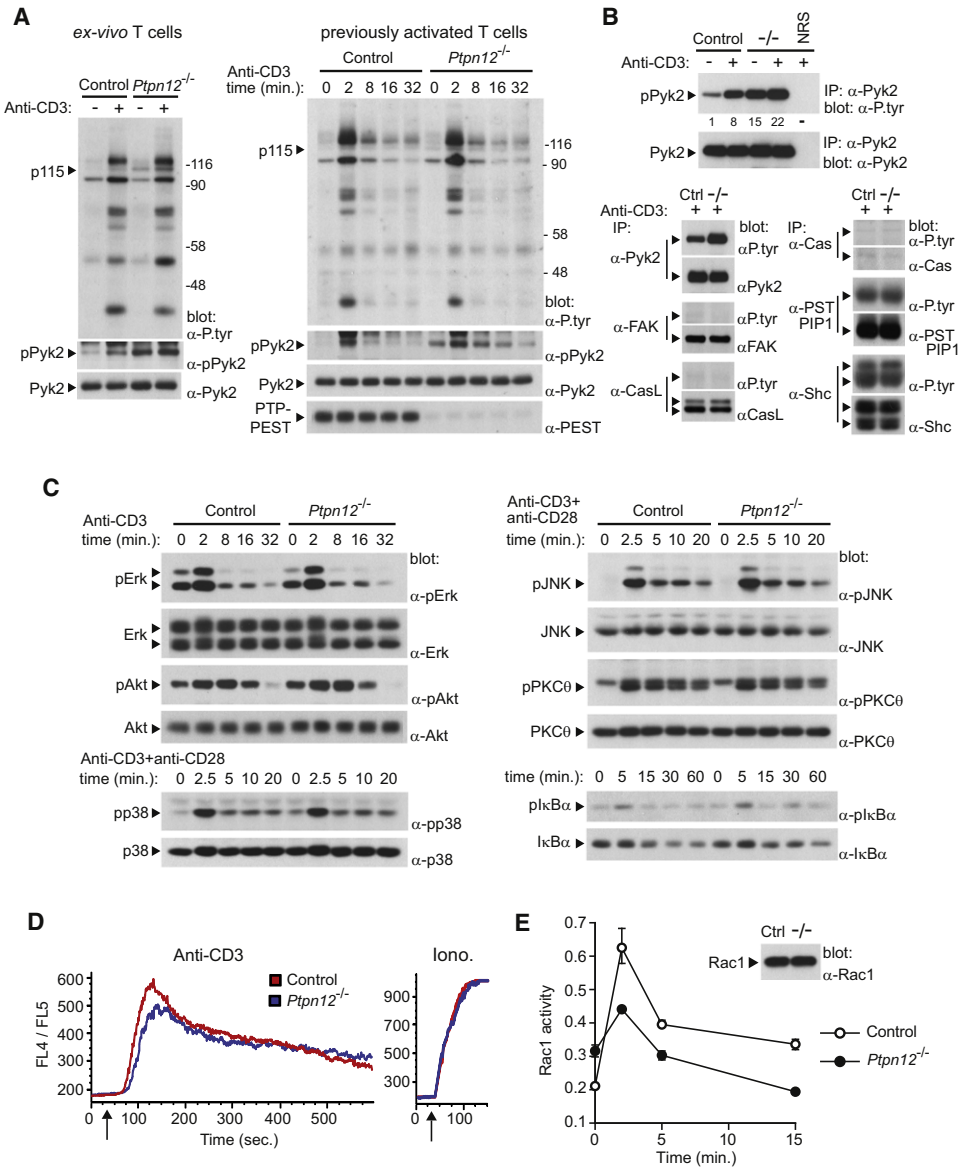


Figure 3. Biochemical Alterations in *Ptpn12*$^{-/-}$ T Cells

(A) Freshly purified (left) or previously activated (right) CD4$^+$ T cells were stimulated with anti-CD3 for 2 min on the left or for the indicated times on the right. Phosphotyrosine (P.tyr)-containing proteins were detected by immunoblotting of total cell lysates with anti (α)-P.tyr. The position of a 115 kilodalton-protein (p115) showing selective hyperphosphorylation in *Ptpn12*$^{-/-}$ T cells is shown on the left. Phosphorylated Pyk2, total Pyk2, and PTP-PEST were also detected by immunoblotting with phospho-specific anti-Pyk2, anti-Pyk2, or anti-PTP-PEST (PEST), respectively. A representative of two (left) or at least ten (right) experiments is shown.

(B–E) Signaling in previously activated CD4$^+$ T cells. As shown in (B), cells were stimulated or not for 2 min with anti-CD3. Polypeptides were recovered by immunoprecipitation and probed by immunoblotting with anti-P.tyr or the specified antibodies. Quantitation of phosphorylated Pyk2 relative to that of the unstimulated control is shown. NRS, normal rabbit serum. A representative of at least five (Pyk2) or two (other substrates) experiments is shown. As shown in (C), cells were stimulated with anti-CD3 or anti-CD3 plus anti-CD28. Activation of Erk, Akt, p38, JNK, PKC-θ, and IκBα was determined by immunoblotting with phospho-specific antibodies. A representative of one to three experiments, depending on the panel, is shown. As shown in (D), cells were loaded with Indo-1 and stimulated with anti-CD3 (left) or ionomycin (Iono; right). Changes in intracellular Ca^{2+} were determined by ascertaining the fluorescence (FL) 4 to FL5 ratio (FL4/FL5), with a BD LSR flow cytometer. An arrow (bottom) indicates when the stimulus was added. A representative of four experiments is shown. As shown in (E), cells were stimulated or not for the indicated periods of time with anti-CD3. Rac1 activity was determined with a Rac1 activation assay kit. Rac1 activity is represented as arbitrary units. Standard deviations of triplicates are shown. Expression of Rac1 was analyzed by Rac1 immunoblotting (insert). A representative of four experiments is shown. See also Figure S3.

stimulation. In contrast, the capacity of *Ptpn12*$^{-/-}$ T cells to form large T cell aggregates upon antigen-MHC restimulation was severely compromised (Figure 4A; Figure S4B). This was true at all times after restimulation. No difference was observed when cells were stimulated with P+I. Activation-induced aggregates are a hallmark of T cell activation. They are formed by

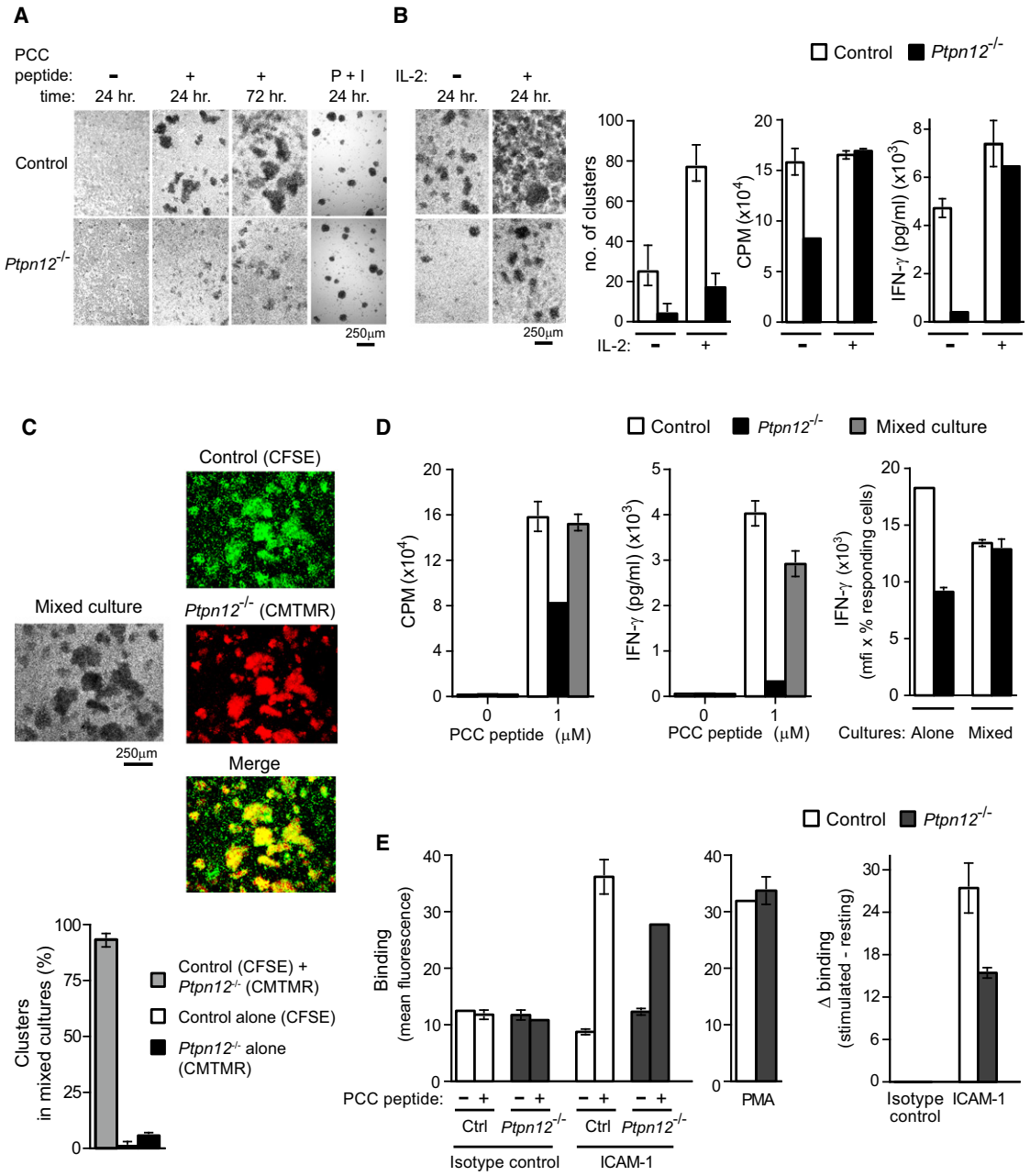


Figure 4. Reduced Homoaggregate Formation by *Ptpn12*$^{-/-}$ T Cells

(A) CD4$^+$ T cells from TCR AND transgenic mice were first activated with anti-CD3 plus anti-CD28. They were then restimulated or not with PCC peptide (1 μM) plus splenocytes or with PMA plus ionomycin (P+I). The formation of cell aggregates was assessed by light microscopy. Cell clusters larger than 8 × 10^{-3} mm^2 (for control or peptide stimulation) or 2 × 10^{-3} mm^2 (for P+I stimulation) were quantitated by counting representative visual fields of 5 mm^2. Representative time points are shown. A more detailed representation of the data is depicted in Figure S4B. Standard deviations of triplicates are shown. A representative of five experiments is shown.

(B) Cells were stimulated for 24 hr and analyzed as detailed in (A), except that IL-2 (100 U/ml) was added or not during restimulation with antigen-MHC. Thymidine incorporation and IFN-γ secretion were also monitored (right). Standard deviations of triplicate values are shown.

(C and D) This experiment was conducted as detailed in (A), except that control cells (labeled with CFSE) and *Ptpn12*$^{-/-}$ T cells (labeled with CMTMR) were stimulated alone (40 × 10^4 cells) or in mixed cultures (20 × 10^4 cells each). A representative of two experiments is shown. As shown in (C), aggregate formation was examined by fluorescence microscopy (top). In mixed cultures, the percentage of clusters containing control cells alone, *Ptpn12*$^{-/-}$ T cells alone or both cell types was quantitated (bottom). A representative of three experiments is shown. As shown in (D), thymidine incorporation and IFN-γ production were monitored. As shown in the middle panel, total IFN-γ production was studied by ELISA. As shown in the right panel, IFN-γ production was analyzed by intracellular staining; production of IFN-γ was calculated by multiplying the mean fluorescence intensity (mfi) of IFN-γ$^+$ cells by the percentage of IFN-γ$^+$ for each cell type in pure or mixed cultures. Standard deviations of triplicates are shown. A representative of two experiments is shown.

T cell homoconjugates and are believed to strengthen T cell activation by favoring exchanges of cytokines, in particular IL-2, between T cells (Sabatos et al., 2008).

To assess whether the decreased aggregate formation was simply due to reduced IL-2 production, we conducted similar experiments in the presence of exogenous IL-2 (Figure 4B). IL-2 only minimally improved aggregate formation by *Ptpn12*$^{-/-}$ T cells, implying that the decreased aggregation was not due to reduced IL-2 secretion. However, addition of IL-2 corrected the thymidine incorporation and IFN-γ secretion defects seen in *Ptpn12*$^{-/-}$ T cells, implying that the stimulatory role of aggregate formation during T cell activation might be bypassed by supplying exogenous IL-2.

To test more directly the contribution of reduced homoaggregate formation to the compromised activation of *Ptpn12*$^{-/-}$ T cells, we studied the ability to rescue the defects of *Ptpn12*$^{-/-}$ T cells by mixing these cells with control T cells (Figures 4C and 4D). Using cells differentially labeled with CFSE or 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR), we found that control T cells rescued aggregate formation by *Ptpn12*$^{-/-}$ T cells (Figure 4C). Considering that the number of control cells in mixed cultures was only half of that in pure cultures, control cells also seemed to correct the thymidine incorporation and IFN-γ secretion defects seen in *Ptpn12*$^{-/-}$ T cells (Figure 4D). To ensure that the latter was the case, single-cell analyses of IFN-γ secretion were also performed with intracellular staining. Compared to pure aggregates, the extents of IFN-γ production by control and *Ptpn12*$^{-/-}$ T cells in mixed aggregates were equivalent.

The integrin leukocyte-function-antigen-1 (LFA-1) and its ligand, intercellular adhesion molecule-1 (ICAM-1), are both expressed on activated T cells and play a critical role in T cell-T cell interactions (Sabatos et al., 2008). Consequently, we examined the impact of PTP-PEST deficiency on the expression and function of these two molecules. PTP-PEST deficiency had no impact on the amounts of LFA-1 and ICAM-1 on activated T cells (Figure S4C). However, it caused a reproducible decrease in the ability of T cells to bind soluble ICAM-1 in response to antigen-MHC, presumably reflecting a decrease in the affinity or avidity of LFA-1 (Figure 4E). This was not the case when cells were stimulated with PMA or when ICAM-1 was immobilized on plastic (Figure 4E; Figure S4D). Immobilization of ICAM-1 on plastic can mask differences in LFA-1 affinity (Kinashi, 2005). Although the magnitude of the changes in soluble ICAM-1 binding may seem small, it was similar to those reported by other groups (Stewart et al., 1996; Baker et al., 2009). Finally, we examined the ability of LFA-1 to mediate intracellular biochemical signals (Figure S4E). Lack of PTP-PEST had no impact on the aptitude of LFA-1 to enhance overall protein tyrosine phosphorylation, Pyk2 tyrosine phosphorylation, and Erk activation, although it augmented baseline Pyk2 tyrosine phosphorylation.

Thus, *Ptpn12*$^{-/-}$ T cells exhibited a strong decrease in T cell homoaggregation, but not in conjugate formation with APCs. This defect correlated with decreased binding of LFA-1 to soluble ICAM-1, presumably reflecting a reduction of the affinity or avidity of LFA-1. No change in LFA-1 expression or signaling capabilities was seen. Enforced homoaggregate formation or the presence of exogenous IL-2 was able to rescue the activation defects seen in *Ptpn12*$^{-/-}$ T cells, suggesting that compromised homoaggregate formation and reduced IL-2 exchanges between aggregated T cells contributed to the diminished responsiveness of *Ptpn12*$^{-/-}$ T cells.

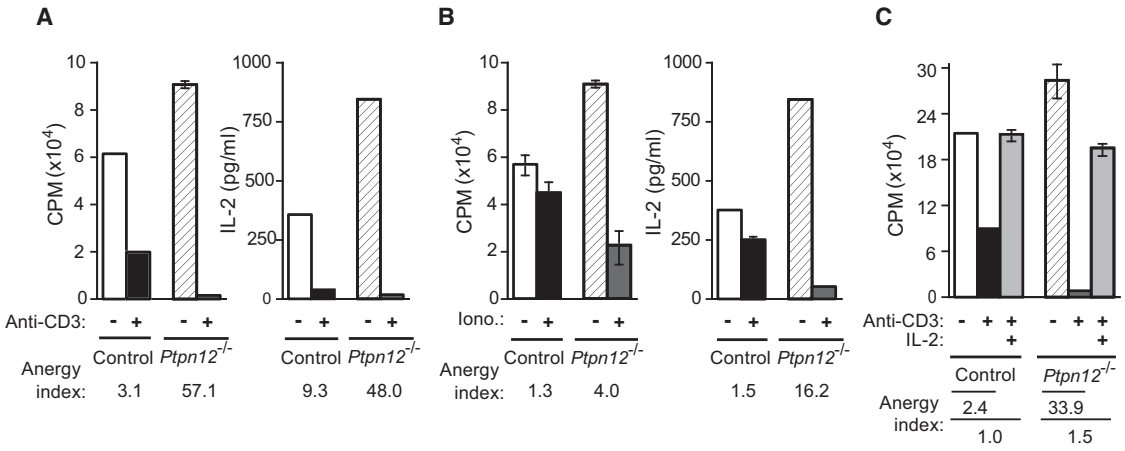
PTP-PEST Inhibits the Induction of Anergy-like States

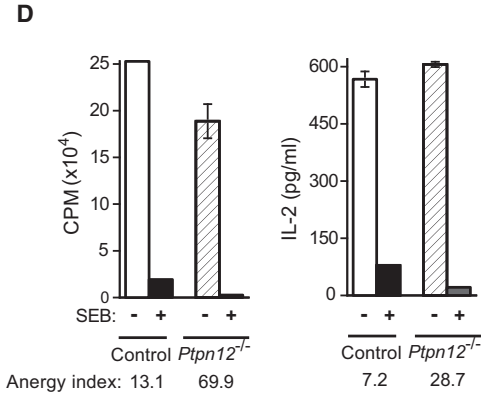
A decrease in responsiveness of previously activated T cells is also seen in T cell anergy, a state of hyporesponsiveness typically produced when initial TCR engagement occurs without adequate costimulation (Choi and Schwartz, 2007). To ascertain whether PTP-PEST influenced sensitivity to anergy-like states, we used two in vitro models: anti-CD3-induced anergy and ionomycin-induced anergy (Figure 5) (Jenkins et al., 1990; Macian et al., 2002). CD4$^+$ T cells were first activated with anti-CD3 plus anti-CD28 and, later, treated or not with anti-CD3 or ionomycin to induce anergy. T cells were then restimulated with anti-CD3 plus anti-CD28 and assayed for thymidine incorporation and IL-2 production. As expected, treatment of control T cells with anti-CD3 (Figure 5A) inhibited subsequent thymidine incorporation and IL-2 production. The "anergy index," defined as the ratio of thymidine incorporation between cells treated with medium and cells exposed to the anergizing regimen, was 3.1. A smaller, albeit reproducible, anergizing effect (index of 1.3) was seen when control cells were treated with ionomycin (Figure 5B). In *Ptpn12*$^{-/-}$ T cells, these indices were increased to 57.1 (~18-fold increase) and 4.0 (~3-fold increase) for anti-CD3- or ionomycin-induced anergy, respectively. Similar effects were seen when IL-2 secretion was measured. As reported in other settings (Choi and Schwartz, 2007), anergy was largely rescued by exogenous IL-2 (Figure 5C).

We also studied the sensitivity of PTP-PEST-deficient mice to anergy in vivo (Figure 5D) (Mittrucker et al., 1996). Mice were injected with the superantigen staphylococcal enterotoxin B (SEB) or phosphate-buffered saline (PBS). After 10 days, CD4$^+$ T cells were restimulated with SEB, and assayed for thymidine incorporation and IL-2 secretion. Control and PTP-PEST-deficient mice showed no difference in the abundance of Vβ8.1/8.2$^+$ CD4$^+$ T cells, which mediate responsiveness to SEB (see the legend of Figure 5). Treatment of control mice with SEB caused an inhibition of thymidine incorporation and IL-2 secretion upon SEB restimulation, in keeping with anergy induction. In PTP-PEST-deficient mice, anergy induction was much stronger. This effect was seen at all concentrations of SEB used for restimulation.

To determine the signaling defects responsible for the enhanced sensitivity of PTP-PEST-deficient mice to anergy-like states, we performed biochemical analyses analogous to those described in Figure 3 (Figure S5). The only observed alteration was an increased Pyk2 tyrosine phosphorylation, both in resting and in anergized T cells (Figure S5A). There was no effect on the extent of nuclear factor of activated T cells (NFAT) and

(E) Previously activated CD4$^+$ T cells were stimulated or not for 30 min, stained with soluble ICAM-1 and assayed by flow cytometry. Staining with an isotype control antibody (human IgG_1) was used as control. The difference in mean fluorescence between stimulated and resting cells (Δ binding) is depicted on the right. Standard deviations of triplicates are shown. A representative of two experiments is shown. See also Figure S4.





Anergy index: proliferation		
SEB (μg/ml)	Control	*Ptpn12*$^{-/-}$
0.37	43.9	93.1
1.1	58.7	170.6
3.3	13.1	69.9
10	20.9	30.3

Anergy index: IL-2 production		
SEB (μg/ml)	Control	*Ptpn12*$^{-/-}$
0.37	10.1	19.0
1.1	12.6	26.9
3.3	7.2	28.7
10	8.1	19.8

Figure 5. Enhanced Susceptibility to T Cell Anergy-like States in PTP-PEST-Deficient Mice

(A–C) CD4$^+$ T cells from the indicated mice were activated with anti-CD3 plus anti-CD28, and subsequently expanded in medium containing IL-2. They were then treated or not with anti-CD3 (1 μg/ml) (A) or ionomycin (Iono; 1 μM) (B) and restimulated with anti-CD3 and anti-CD28. In (C), recombinant IL-2 (100 U/ml) was added during the final restimulation. Proliferation was determined by thymidine incorporation, and IL-2 secretion was assessed by ELISA. The anergy index is the ratio of thymidine incorporation or IL-2 secretion between untreated and anti-CD3- or ionomycin-treated cells. Standard deviations of triplicates are shown. A representative of three experiments is shown.

(D) Mice were injected i.p. with *Staphylococcus aureus* enterotoxin B (SEB; 100 μg) or PBS alone. After 10 days, splenic CD4$^+$ T cells were isolated and incubated for 4.5 days with SEB (0.37–10 μg/ml) and splenocytes. Thymidine incorporation and IL-2 secretion were monitored. The anergy index is the ratio of thymidine incorporation or IL-2 secretion between PBS-injected and SEB-injected mice. The anergy indices for restimulation with 3.3 μg/ml of SEB are shown on the left. Standard deviations of triplicate values are depicted. The values for all SEB concentrations are represented on the right. The proportions of Vβ8.1/8.2$^+$ cells in CD4$^+$ T cells were: PBS-treated mice: control, 16.0%; *Ptpn12*$^{-/-}$, 17.2%; SEB-treated: control, 11.5%; *Ptpn12*$^{-/-}$, 11.4%. A representative of four experiments is shown. See also Figure S5.

activator protein-1 (AP-1) activation during anergy induction (Figure S5B). Consequently, PTP-PEST was inhibiting the induction of anergy-like states in T cells, and this effect specifically correlated with dephosphorylation of Pyk2.

Suppression of Pyk2 Reduces Anergy-like States

The selective hyperphosphorylation of Pyk2 in *Ptpn12*$^{-/-}$ T cells suggested that deregulation of Pyk2 was altering their responsiveness. To test this idea, we examined the impact of suppressing Pyk2 function on T cell anergy, utilizing three complementary approaches (Figure 6; Figure S6). First, we examined the effect of downregulating Pyk2 expression by using small interfering (si) RNAs (Figures 6A and 6B). T cells were infected with retroviruses encoding Pyk2-specific or nonspecific siRNAs. Cells expressing Pyk2-specific siRNAs exhibited a partial downregulation of Pyk2 expression (Figure 6A). Furthermore, they had a reduced sensitivity to anti-CD3-induced anergy (Figure 6B). This was true both for control T cells and for *Ptpn12*$^{-/-}$ T cells.

Second, we used a pharmacological inhibitor of Pyk2, PF-562,271 (Figures S6A and S6B) (Bagi et al., 2008; Roberts et al., 2008). This compound efficiently inhibits Pyk2 and FAK, but not Fyn and Lck. Because we detected very little if any tyrosine phosphorylation of FAK in our studies, we assumed that the effect of the inhibitor was not mediated by FAK. PF-562,271 suppressed tyrosine phosphorylation of Pyk2, but not overall protein tyrosine phosphorylation, in resting and activated T cells (Figure S6A). Moreover, it prevented the ability of anti-CD3 to induce anergy, both in control T cells and in *Ptpn12*$^{-/-}$ T cells (Figure S6B). It is possible that the stronger

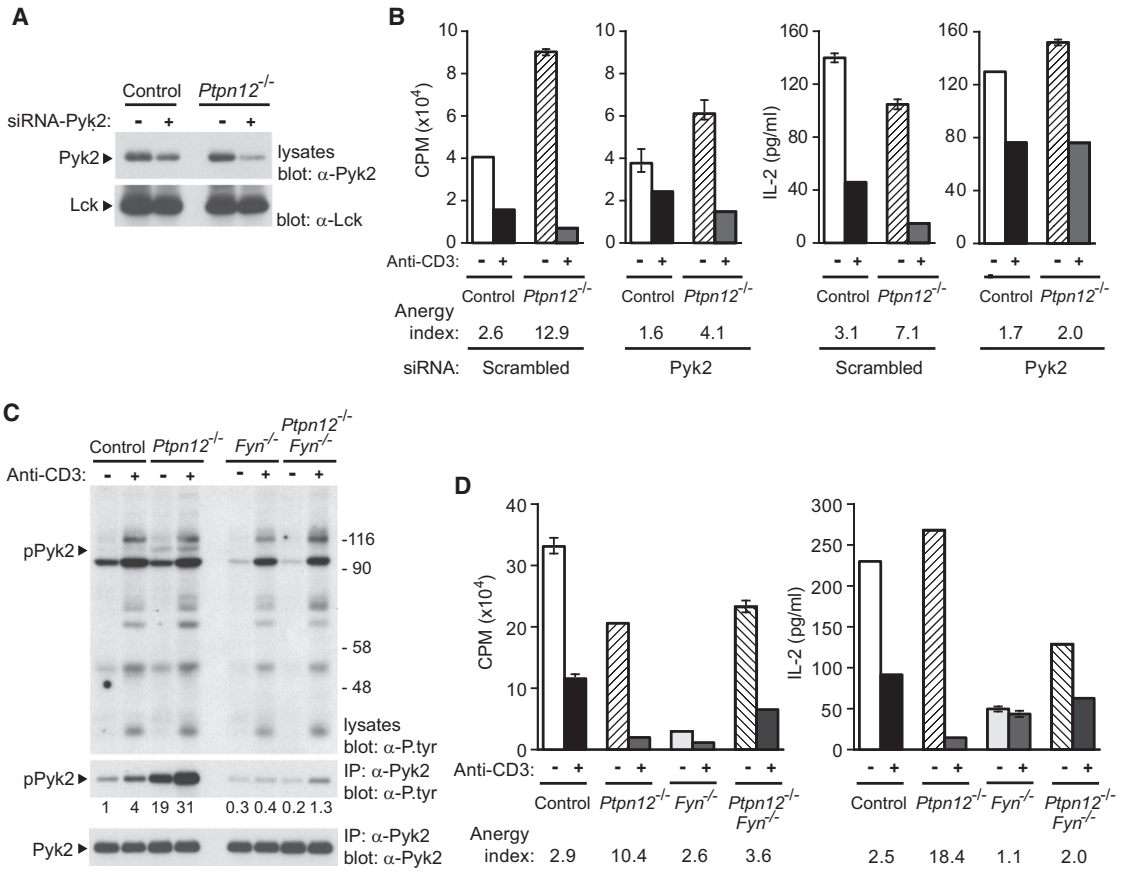


Figure 6. Impact of Pyk2 Inhibition on Anergy-like States

(A and B) Previously activated CD4$^+$ T cells were infected with retroviruses encoding green fluorescent protein (GFP) and Pyk2-specific or irrelevant ("Scrambled") small interfering (si) RNAs. After sorting GFP$^+$ cells, cells were analyzed by immunoblotting with anti-Pyk2 (A) or for anti-CD3-induced anergy as detailed for Figure 5 (B).

(C and D) Previously activated CD4$^+$ T cells from the indicated mice were stimulated or not for 2.5 min with anti-CD3 and various signals were tested as detailed for Figure 3 (C). They were also analyzed for anti-CD3-induced anergy (D). All panels are representative of two experiments. See also Figure S6.

rescue effected by the pharmacological inhibitor, in comparison to the Pyk2 siRNAs, was due to a more complete inhibition of Pyk2.

Third, we examined the effect of lack of Fyn, the PTK required for TCR-triggered tyrosine phosphorylation of Pyk2 (Qian et al., 1997), by breeding PTP-PEST-deficient mice with *Fyn*$^{-/-}$ mice (Figure 6C,D; Figure S6C). Fyn has been shown to promote T cell anergy in some systems (Utting et al., 2001; Davidson et al., 2007), thus potentially opposing the role of PTP-PEST in this process. *Ptpn12*$^{-/-}$*Fyn*$^{-/-}$ T cells lacked both PTP-PEST and Fyn (Figure S6C). They exhibited a marked correction of the elevated Pyk2 tyrosine phosphorylation observed in *Ptpn12*$^{-/-}$ T cells and, in keeping with the role of Fyn in TCR signaling, a decrease in TCR-triggered protein tyrosine phosphorylation (Figure 6C). More importantly, they showed a markedly reduced sensitivity to anergy (Figure 6D).

Thus, PTP-PEST deficiency enhanced the susceptibility of T cells to anergy-like states at least in part by causing a deregulation of Pyk2. Lack of Fyn corrected the enhanced Pyk2 tyrosine phosphorylation and anergy induction in *Ptpn12*$^{-/-}$ T cells, suggesting that PTP-PEST, Pyk2, and Fyn are part of a common pathway involved in anergy regulation.

PTP-PEST-Deficient Mice Are Less Susceptible to a T Cell-Dependent Immunopathology

The decrease in secondary T cell responses seen in PTP-PEST-deficient mice suggested that these mice might be less susceptible to certain autoimmune diseases. To address this issue, we examined their susceptibility to experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (Figure 7; Figure S7) (Zhong et al., 2002). In this model, activated CD4$^+$ T cells (T helper 1 [Th1] and Th17 cells) are generated in peripheral lymphoid organs after immunization with a MOG peptide (MOG_{35-55}). MOG-specific T cells then migrate to the central nervous system (CNS), where they are reactivated by endogenous MOG and cause neurological damage.

Compared to control mice, PTP-PEST-deficient mice were much less susceptible to EAE (Figure 7A). Both disease frequency and disease severity were lower. This reduction was not due to a defect in the initial immune response to MOG, given that PTP-PEST-deficient mice did not exhibit a marked defect in the production of MOG antibodies (Figure 7B). To verify that the reduced susceptibility to EAE was not simply due to a defect in migration of *Ptpn12*$^{-/-}$ T cells through the blood-brain barrier, we tested secondary MOG-specific CD4$^+$ T cell responses in

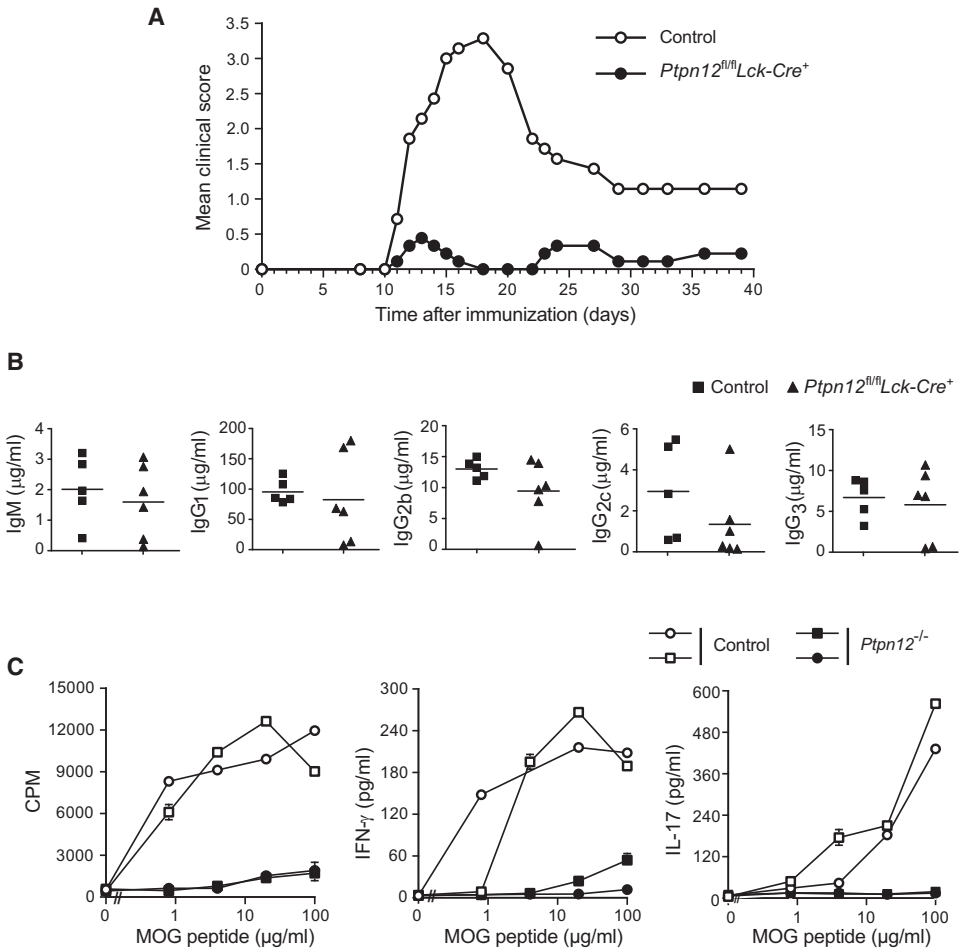


Figure 7. Diminished Sensitivity to Experimental Autoimmune Encephalomyelitis in PTP-PEST-Deficient Mice

(A) Mice (n = 7–9 in each group) from the indicated strains were immunized with myelin oligodendrocyte (MOG) peptide 35-55 in adjuvant. After an additional treatment with pertussis toxin, mice were observed daily for neurological deficits as detailed in the Experimental Procedures. The mean values of the clinical scores for the different groups of mice are shown. A representative of four experiments is shown.

(B) MOG-specific immunoglobulins (Ig) were detected in the serum of immunized mice (day 40) by ELISA. Symbols represent individual mice. Average values are shown as horizontal bars. In all cases, p values were nonsignificant (>0.05). A representative of two experiments is shown.

(C) Six weeks after MOG immunization, $CD4^+$ T cells were purified from spleen and restimulated with MOG peptide plus splenocytes. Two mice from each group are depicted. After 4–5 days, proliferation was determined by measuring thymidine incorporation, whereas cytokine secretion was assessed by ELISA. Standard deviations of triplicates are shown. A representative of five experiments is shown. See also Figure S7.

peripheral lymphoid tissues, by using the in vitro restimulation assay (Figure 7C). *Ptpn12*$^{-/-}$ splenic T cells exhibited a severely compromised ability to proliferate and secrete cytokines in response to MOG restimulation. In contrast, responsiveness to P+I was normal (Figure S7). Therefore, PTP-PEST deficiency reduced the susceptibility to EAE, and this effect correlated with a systemic deficit in secondary T cell responses.

DISCUSSION

Our studies revealed that loss of PTP-PEST, like that of LYP (Hasegawa et al., 2004), had no appreciable impact on thymocyte development, T cell differentiation, and primary T cell responses. The lack of a marked effect of PTP-PEST deficiency on primary T cell responses was in contrast to the results of earlier overexpression studies, which suggested a strong inhibitory role of PTP-PEST during T cell activation (Badour et al., 2004; Arimura et al., 2008). Possibly, the inhibitory effect of PTP-PEST seen in these previous studies was due to nonphysiological consequences of overexpression. Alternatively, the remaining PTP-PEST expression (<5% of control) in our *Ptpn12*$^{-/-}$ T cells was perhaps sufficient to offset any alterations of primary responses.

In contrast to primary T cell responses, however, PTP-PEST deficiency had a prominent impact on secondary responses. Previously activated *Ptpn12*$^{-/-}$ $CD4^+$ T cells expressing a transgenic TCR exhibited a decrease in cytokine secretion and proliferation upon restimulation in vitro. Similarly, after immunization of PTP-PEST-deficient mice with OVA and adjuvant, T cells demonstrated severely reduced thymidine incorporation and cytokine release when restimulated with OVA in vitro. There was no evidence that the defect in responsiveness of *Ptpn12*$^{-/-}$ T cells was caused by augmented Treg cell numbers or activity.

In some ways, the decreased responsiveness of *Ptpn12*$^{-/-}$ T cells to secondary stimulation was reminiscent of anergy. In support of this similarity, *Ptpn12*$^{-/-}$ T cells were also more prone to develop anergy-like states. Combined with the results outlined above, this finding implied that PTP-PEST deficiency was compromising the ability of activated T cells to respond to repeated stimulation. This phenotype is diametrically opposed to that of LYP deficiency, which augments the responsiveness of previously activated T cells (Hasegawa et al., 2004; Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999).

Ptpn12$^{-/-}$ T cells exhibited an increase in tyrosine phosphorylation of Pyk2, but not of other proteins including known PTP-PEST substrates. Coupled with our finding that PTP-PEST was able to dephosphorylate Pyk2 in vitro, these data established that Pyk2 was a direct substrate of PTP-PEST in T cells. PTP-PEST has previously been shown to interact with Pyk2 by way of the adaptor paxillin (Jamieson et al., 2005; Shen et al., 1998; Lyons et al., 2001; Davidson and Veillette, 2001). To ascertain whether Pyk2 deregulation explained the abnormal responsiveness of *Ptpn12*$^{-/-}$ T cells, we tested the influence of Pyk2 inhibition on the susceptibility to anergy-like states. Expression of Pyk2-specific siRNAs or treatment of T cells with a Pyk2 inhibitor was efficient at preventing anergy. These protective effects were observed not only in *Ptpn12*$^{-/-}$, but also in normal T cells. Moreover, removal of Fyn, the PTK that phosphorylates Pyk2 in T cells, corrected the augmented anergy susceptibility of *Ptpn12*$^{-/-}$ T cells. Hence, deregulated Pyk2 was responsible at least in part for the increased propensity of *Ptpn12*$^{-/-}$ T cells to become anergized.

Whereas *Ptpn12*$^{-/-}$ T cells exhibited normal activation of most TCR-regulated signals other than Pyk2, they had increased baseline activity of Rac1, coupled to decreased TCR-triggered activation of Rac1. Because Rac1 is a downstream effector of Pyk2 (Gismondi et al., 2003), deregulation of Rac1 was probably consequential to altered Pyk2 function. In agreement with the involvement of PTP-PEST, Pyk2 and Rac1 in adhesion, *Ptpn12*$^{-/-}$ T cells also had altered adhesive properties. Although they had no defect in the ability to adhere to antigen-presenting B cells, they had a severe deficiency in the ability to form homotypic aggregates during T cell activation. T cell homoaggregates promote T cell activation and are highly dependent on LFA-1-ICAM-1 interactions (Sabatos et al., 2008). Consistently, we found that LFA-1 on *Ptpn12*$^{-/-}$ T cells had a reduced ability to bind soluble ICAM-1, suggesting that the capacity of LFA-1 on one T cell to bind ICAM-1 on another T cell might be reduced. Importantly, addition of exogenous IL-2 or control T cells restored thymidine incorporation and cytokine production by *Ptpn12*$^{-/-}$ T cells. Therefore, PTP-PEST may promote secondary T cell responses by enhancing LFA-1-dependent T cell homoaggregate formation, possibly by controlling Pyk2 and Rac1. As suggested (Sabatos et al., 2008), this could in turn favor exchanges of IL-2 between aggregated cells, thereby augmenting T cell proliferation and effector responses. Whether or not a similar mechanism is involved in protecting against anergy remains to be examined.

The finding that PTP-PEST deficiency compromised the responsiveness of previously activated, but not of naive, T cells was surprising. The deleterious influence of PTP-PEST deficiency may necessitate changes in another cellular protein, perhaps implicated in Pyk2-mediated functions, that occur only after a first round of T cell activation. Alternatively, the activation-promoting effect of T cell homoaggregation may occur primarily during secondary T cell activation. Although the function of LYP in T cells is qualitatively opposite to that of PTP-PEST, a related situation exists for LYP. LYP-deficient mice exhibit altered secondary, but not primary, T cell responses (Hasegawa et al., 2004; Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999).

T cell anergy and anergy-like states are complex processes that can be triggered in a variety of experimental settings (Choi and Schwartz, 2007). They have been linked to a range of signaling abnormalities, ranging from a global decrease in TCR-triggered protein tyrosine phosphorylation to a more limited effect on Erk activation. It is believed that anergy and anergy-like states are the result of an imbalance in T cell signaling, in which TCR signaling is not accompanied by proportional signaling through costimulatory molecules like CD28. The findings that PTP-PEST deficiency predisposed T cells to anergy-like states and that inhibition of Pyk2, a PTP-PEST substrate, prevented anergy-like states provided compelling evidence that PTP-PEST and Pyk2 are also involved in the regulation of anergy. Interestingly, though, this activity of PTP-PEST and Pyk2 appeared to be independent of classical anergy mechanisms, given that activation of NFAT and AP-1 was not affected in *Ptpn12*$^{-/-}$ T cells. Therefore, PTP-PEST and Pyk2 may be implicated in an alternative anergy-regulating mechanism. This mechanism may also involve Fyn, given that elimination of Fyn alleviated the increased Pyk2 tyrosine phosphorylation and the enhanced sensitivity to anergy-like states observed in *Ptpn12*$^{-/-}$ T cells. Therefore, PTP-PEST, Pyk2 and Fyn may be part of a common pathway that influences anergy induction, independently of deregulation of NFAT and AP-1. A recent report also linked Pyk2 to an NFAT-AP-1-independent mechanism of inhibition in macrophages (Wang et al., 2010).

Mice lacking PTP-PEST in T cells were much more resistant to EAE, a CD4^{+} T cell-mediated immunopathology. This defect correlated with a reduction of secondary T cell responses to the immunizing antigen in in vitro restimulation assays and, thus, was likely due to decreased reactivation in the CNS of MOG-specific T cells generated in the periphery. Given the role of PTP-PEST in migration in other cell types (Larsen et al., 2003), it is possible that a defect in T cell migration across the blood-brain barrier also participated in this phenotype. Although future studies are warranted to address the role of PTP-PEST in T cell migration, alterations of T cell migration were unlikely to explain the decreased sensitivity of PTP-PEST-deficient mice to EAE considering that the responsiveness of splenic T cells to MOG restimulation was also severely compromised. Therefore, the defect in T cell responsiveness was systemic rather than restricted to the CNS.

In summary, our data showed that PTP-PEST deficiency in T cells resulted in compromised secondary responses, enhanced susceptibility to anergy-like states, and diminished vulnerability to autoimmunity. Consequently, PTP-PEST carries out a critical and unique function in T cells, further illustrating the important role of PTPs in T cell regulation. This function is diametrically opposite to that of LYP (Hasegawa et al., 2004; Cloutier and Veillette, 1999; Gjorloff-Wingren et al., 1999) and seems to be due to the ability of PTP-PEST to regulate Pyk2, a Fyn substrate. Our results also suggested that modulation of PTP-PEST

function may be of value for the treatment of some human diseases. The finding that PTP-PEST deficiency diminished the susceptibility to EAE raised the possibility that inhibition of PTP-PEST or PTP-PEST-regulated pathways may help treat multiple sclerosis and other autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

The conditional allele of *Ptpn12* was generated as described in Supplemental Experimental Procedures. *Ptpn12*$^{fl/+}$ mice were crossed with C57BL/6 mice for at least eight generations and bred with transgenic mice expressing the Cre recombinase under the control of the *Cd4* or *Lck* promoter (from Taconic, Hudson, NY). In some experiments, mice were crossed with class II MHC-restricted PCC-specific TCR transgenic mice AND or class II MHC-restricted OVA-specific TCR transgenic mice OT-II (obtained from Taconic) (Barnden et al., 1998; Kaye et al., 1989). C57BL/6 mice were obtained from Harlan Laboratories (Chicago, IL), whereas B10.BR mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal experimentation was approved by the IRCM Animal Care Committee and done in accordance with the regulations of the Canadian Council for Animal Care.

T Cell Activation

For generating previously activated T cells, purified CD4$^+$ T cells were stimulated with plate-bound anti-CD3 mAb 145-2C11 (3 μg/ml) and soluble anti-CD28 mAb 37-51 (1 μg/ml) for 2 days and then expanded for 3 days in IL-2 (50 U/ml)-containing medium. For activation, freshly isolated or previously activated T cells were stimulated for 48 hr with plate-coated anti-CD3 (1–10 μg/ml), with or without soluble anti-CD28 (1 μg/ml). For TCR AND mice, CD4$^+$ T cells were stimulated for 6 days with the PCC peptide 88-104 (0.1–10 μM), in the presence of irradiated splenocytes from B10.BR mice. For TCR OT-II mice, CD4$^+$ T cells were stimulated for 2 days with the OVA peptide 323-339 (0.04–25 μM), in the presence of irradiated splenocytes from C57BL/6 mice. As control, cells were stimulated with PMA (100 ng/ml) and ionomycin (1 μM). After stimulation, proliferation was measured by assaying for [^{3}H]-thymidine incorporation, whereas cytokine production was revealed by ELISA (R&D Systems, Minneapolis, MN). All assays were done in triplicate.

Immunization with Ovalbumin

Mice were immunized in the foot pad with OVA protein (100 μg in 25 μl of PBS; Sigma-Aldrich, St. Louis, MO) in the presence of an equal volume of complete Freund adjuvant (CFA; Sigma-Aldrich). After 9 days, CD4$^+$ T cells were isolated from popliteal lymph nodes and restimulated in vitro for 4–5 days in the presence of OVA (50–200 μg/ml) and irradiated splenocytes, or for 2 days with P+I. Proliferation and cytokine production were determined. OVA-specific CD4$^+$ T cells were numerated by staining with phycoerythrin (PE)-labeled class II MHC I-A(b) tetramers loaded with OVA peptide 323-339 or an irrelevant peptide (human class II-associated invariant chain peptide 103-117) (provided by the NIH Tetramer Facility, Atlanta, GA). Alternatively, T cells were stimulated for 5 hr with PMA (50 ng/ml) and ionomycin (800 ng/ml), and IFN-γ production was assessed by intracellular staining, in accordance with the protocol of the manufacturer (BD Biosciences, Mississauga, ON, Canada). For evaluating suppressor activity, CD4$^+$ T cells from OVA-immunized PTP-PEST-deficient mice were added to those from OVA-immunized control mice at the indicated ratios, prior to restimulation with OVA and irradiated splenocytes. Production of OVA antibodies was determined by ELISA as detailed in Supplemental Experimental Procedures.

T Cell-Dependent Antibody Production

Mice (6–12 weeks old) were injected intraperitoneally (i.p.) with TNP(12)-CGG (100 μg in 100 μl of PBS; from Biosearch Technologies, Novato, CA), in the presence of an equivalent volume of alum (Pierce, Rockford, IL). TNP antibodies were measured in the serum by ELISA as detailed in the Supplemental Experimental Procedures.

Anergy

For induction of anergy in vitro with anti-CD3 or ionomycin, CD4$^+$ T cells were first activated for 48 hr with anti-CD3 and anti-CD28 and then expanded for 3 days in IL-2. After this, anergy was induced by treating cells for 16 hr with plate-bound anti-CD3 (1 μg/ml) or ionomycin (1 μM). For anergy triggered with anti-CD3, cells were then washed and rested for 30 hr. They were later restimulated for 48 hr with anti-CD3 (0.037, 0.11, or 0.33 μg/ml) and anti-CD28 (1 μg/ml). For anergy induced by ionomycin, cells were immediately restimulated with anti-CD3 plus anti-CD28. Thymidine incorporation and IL-2 secretion were measured. For induction of anergy in vivo, mice were injected i.p. with *Staphylococcus aureus* enterotoxin B (SEB) (Sigma-Aldrich; 100 μg in 100 μl of PBS) or with PBS alone. After 10 days, purified splenic CD4$^+$ T cells were stimulated for 4.5 days with various concentrations of SEB (0.37–10 μg/ml), in the presence of irradiated splenocytes. Thymidine incorporation and IL-2 secretion were measured. The percentage of Vβ8.1/8.2$^+$ cells in purified CD4$^+$ T cells was determined by staining with TCR Vβ8.1/8.2-specific mAb MR5-2.

Binding to Soluble ICAM-1

Previously activated CD4$^+$ TCR AND T cells were left unstimulated or stimulated for 30 min at 37°C with purified B cells, loaded or not with PCC peptide (5 μM), or with PMA (50 ng/ml). After washing, cells were incubated for 30 min at room temperature with mouse ICAM-1/Fc chimera (R&D Systems, Minneapolis, MN) prelabeled with Alexa Fluor 647 (Molecular Probes, Eugene, OR). Cells were washed and ICAM-1 binding was evaluated by flow cytometry. Alexa Fluor 647-labeled human IgG_1 was used as isotype control.

Pyk2 Inhibition

For downregulation of Pyk2 expression with siRNA, previously activated T cells were infected with the retroviral vector pSUPER.retro.neo+gfp (OligoEngine, Seattle, WA) containing the sequences detailed in Supplemental Experimental Procedures. Infected cells were purified by sorting GFP-positive cells. After 1–2 days, they were processed for biochemical studies or anergy induction with anti-CD3.

EAE

Mice were injected subcutaneously with 200 μl of an emulsion containing 200 μg of MOG peptide 35-55 in CFA plus 300 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). 24 and 72 hr after the immunization, mice were also injected i.p. with 300 ng of pertussis toxin in 0.5 ml of PBS. They were then observed daily and scored for neurological deficits as follows: 0, no clinical signs; 1, loss of tail tonicity; 2, flaccid tail; 3, hind leg paralysis; 4, hind leg paralysis with hind body paresis; 5, hind and fore leg paralysis; and 6, death. After 6 weeks, CD4$^+$ T cells were purified from spleen and restimulated in vitro with various concentrations of MOG peptide plus irradiated splenocytes, or with P+I, as detailed above for OVA immunization. Production of MOG antibodies was also measured.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2010.08.001.

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