Cyclin kinase inhibitor p21: a mediator of immune tolerance: direct and indirect evidence

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(Words: Abstract 250; main text excluding references and figures 2999)

Running Title: p21 and immune tolerance

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Immune tolerance considered to be a holy grail for long-term graft survival of organ transplants. However, the current methodologies, agents and strategies have failed to achieve measurable immune tolerance. Therefore, there is a need to discover alternate mediators and pathways that mediate the events leading to immune tolerance. Since aberrant proliferation is considered to be the barrier to immune tolerance, molecules that control cell cycle progression may be the key components in this process. Cyclin kinase inhibitor p21 is such a molecule, which is potent regulator of the events of cellular proliferation and apoptosis[1, 2], the key determinants of immune tolerance. We have shown that cyclins and p21 play reciprocal role in immune activation and inflammation[3]. Additionally, our studies have demonstrated that *in vitro* and *in vivo* over-expression of p21 renders lymphocytes less responsive to mitogenic stimuli, decrease inflammation and prolong graft survival [4, 5]. We have also reported that recombinant p21 protein interacts *in vivo* with transcription factors, inhibits lymphocyte proliferation and inflammation [6]. In this study, we demonstrate that p21 by virtue of its properties similar to CD4+CD25+ T regulator cells may be a key component of the process of immune tolerance.

The results from wild type [n =9] and $p21^{-/-}$ mice [n = 9] are shown in **Figure 1 A**. The splenocytes from $p21^{-/-}$ mice proliferated significantly [$p<0.01$] more compared to wild -type mice. We also studied the effect of cyclosporine [CsA], on the proliferation of lymphocytes from wild type and $p21^{-/-}$ mice. To determine if the increased proliferation of $p21^{-/-}$ mice is the direct effect of p21 deficiency, the expression of p21 was induced in $p21^{-/-}$ mice; using our previously described procedure[4]. We injected mice with either p21 sense or empty vector plasmid DNA [intramuscularly] two times at an interval of 5 days. Mice were sacrificed 7 and 21 days after the last injection [n=4 in each group]. **Figure**

1C demonstrates that the transfection of p21 in $p21^{-/-}$ mice resulted in a significantly decreased proliferation in response to anti-CD3, although more inhibition after 7 days compared to 21 days. Since there is a parallel between the expression of cyclins and pro-inflammatory genes[3] and FoxP3 is an indicator of tolerance associated with decreased lymphocyte proliferation, we also studied expression of cyclin G and pro-inflammatory cytokines IFN-γ and TNF-α mRNA expression and its effect on FoxP3 mRNA expression. Lymphocytes from $p21^{-/-}$ mice with and without p21 overexpression were activated with anti-CD3. RNA were prepared, reverse transcribed to cDNA and amplified for cyclin G, IFN-γ, TNF-α and FoxP3 mRNA by Real Time-PCR. The mRNA expression of cyclin G [$p<0.02$], IFN-γ [$p<0.05$] and TNF-α [$p<0.04$] decreased significantly in anti-CD3 stimulated splenocytes from p21 transfected $p21^{-/-}$ mice compared to the $p21^{-/-}$ mice transfected with the control plasmid DNA. However, a significant increase in FoxP3 mRNA was observed. These results [**Figure 1D**] demonstrate that the expression of p21 in $p21^{-/-}$ mice inhibit the expression of cyclins and of pro-inflammatory cytokines and promote FoxP3 expression, suggesting shifting a balance toward CD4+CD25+ T regulatory cells.

The above described results demonstrate that p21 deficiency results in increased proliferation of lymphocytes, mRNA expression of pro-inflammatory cytokines and therefore, we determined if this effect is due to the imbalance of CD4+CD25+ and CD4+CD25- cells. We quantified CD4+CD25+ and CD4+CD25- T cells in spleen cells obtained from wild -type and $p21^{-/-}$ mice. The fluocytometric analysis demonstrated that in CD4+CD25+ cells comprised to 14.5± 1.6 in wild type mice and 2.6 ± 0.8 percent of total cells in $p21^{-/-}$mice, which were significantly [$p<0.002$] lower in these mice [**Figure**

2A]. These results suggest that lack of p21 results in increased lymphocyte proliferation due to diminished number of CD4+CD25+ T regulatory cells. Since FoxP3 mRNA expression is expressed only on CD4+CD25+ but not CD4+CD25- T cells, we determined and quantified mRNA expression of FoxP3 in these cells isolated from wild type and $p21^{-/-}$ mice. The results from a representative of three $p21^{-/-}$ and wild type mice demonstrates that FoxP3 expression was observed only in splenocytes from wild type and not from $p21^{-/-}$ mice [**Figure 2 B**]. These results suggest that $p21^{-/-}$ mice have significantly lower number of CD4+CD25+ T regulatory cells that was reflected by FoxP3 mRNA in splenocytes. To confirm these results, using Real Time PCR analysis, we quantified FoxP3 mRNA in CD4+ T cell subsets; CD4+CD25+ and CD4+CD25- T cells. The results [**Figure 2C**] demonstrate that amplification was observed only in CD4+CD23+ but not in CD4+CD25- T cells. Identical amplification of β-actin mRNA was observed in these cDNA samples. We also calculated fold expression in these CD4+ T cell subsets using β-actin as reference gene and a in built formula 2[[Ct/beta-actin − Ct/gene of interest]].. The results shown in **Figure 2D** demonstrate that CD+CD25+ expressed about 40 fold increased FoxP3 mRNA compared to CD4+CD25- T cells in a single sample, mean ± SEM of relative fold expression from 6 such experiments is shown in **Figure 2E,** indicate a significantly higher expression of FoxP3 mRNA in CD4+CD25+ T cells from wild type mice compared to $p21^{-/-}$ mice. These results confirm the fewer number of FoxP3+ CD4+CD25+ T cells in $p21^{-/-}$ mice compared to wild type mice. More significantly, co-cultured CD4+CD25+ cells from wild type and $p21^{-/-}$ mice exhibited increased FoxP3 mRNA in an autologous MLR assay.

These results described above suggest that the characteristics of p21 deficient cells are similar to CD4+CD25- T cells with respect to the deficiency of FoxP3 mRNA To compare and contrast the immunological characteristics of p21 deficient and p21 expressing with CD4+CD25+/- T regulatory cells., experiments were planned. We compared n the proliferation of purified CD4+CD25- and CD4+CD25+ T cells [n=6]. The difference in proliferation [counts per minute] among CD4+CD25+ [15236 ± 1190] and CD4+CD25- [50317 ± 974] T-cells was highly significant [$p<0.003$]. These results [**Figure 3A**] are similar to those observed with splenocytes from wild type and $p21^{-/-}$ mice [**Figure 1A**], suggesting that the immunological response of p21 expressing, CD4+CD25+ and p21 deficient, CD4+CD25- cells to mitogenic stimuli is similar. Similar to p21 deficient lymphocytes [**Figure 1B**], anti-CD3 activated CD4+CD25- T cells were not responsive to the inhibitory effects of CsA [**Figure 3B**]. This similarity can only be explained due to the inability of CsA to induce p21 expression in both p21 deficient and CD4+CD25- T cells, additionally, it also suggest an interaction between p21 and FoxP3. We performed experiments on CD4+CD25+ and CD4+CD25- T cells to understand if p21 plays any role in the function of these cells. We analyzed TGF-β and p21 mRNA in CD4+CD25+ and CD4+CD25- T cells activated with and without CsA. The results demonstrate that CsA induced TGF-β and p21 mRNA expression in CD4+CD25+ T cells [**Figure 3C,**] but not in CD4+ CD25- T cells [**Figure 3D**]. These results suggest that TGF-β and p21 play a significant role in the function of CD4+CD25+ T cells. Therefore, these results suggest that the inability of these cells to induce expression of either TGF-β or p21, the most powerful anti-proliferative molecules may contribute to heightened proliferative response of CD4+CD25- and $p21^{-/-}$ lymphocytes.

We also performed studies to understand if similar to the reported inhibition of proliferation of CD4+CD25t- T cells by CD4+CD25+ T cells, wild type [p21 expressing] T cells will inhibit proliferation of T lymphocytes from $p21^{-/-}$ mice. We performed an autologous MLR assay using lymphocytes from either WT or p21 deficient cells as responder or stimulators. The results were compared with CD4+CD25+ and CD4+CD25- T lymphocytes. The results shown in the **Figure 4A** demonstrate that the proliferation of CD4+CD25+/ CD4+CD25- T cells is in the median range of these cells alone, indicating that CD4+CD25+ induce inhibitory response in CD4+CD25- T cells. These results were complemented by an almost median expression of T regulatory cells specific gene expression for Foxp3, ILT3 and GITR [**Figure 4B**]. Results in **Figure 4C** demonstrate that in an autologous MLR assay, an inhibition of proliferation lymphocytes from $p21^{-/-}$ mice was observed when lymphocytes from WT mice that express p21 were used as stimulators. These results are similar to those observed with CD4+CD25+ and CD4+CD25- T cells and identical pattern of FoxP3 mRNA was observed in p21 expressing, $p21^{-/-}$ and mixture of these cells after MLR assay [**Figures 4A and 4C**]. FoxP3 mRNA in these cells confirms the immunological response of these subsets of lymphocytes from wild type and $p21^{-/-}$ mice in autologous MLR assay [**Figure 4D**].

The results from this study demonstrate that the immunological responses of p21 deficient lymphocytes and CD4+CD25- T regulatory lymphocytes are similar. Our earlier studies have demonstrated that *in vitro* and *in vivo* over-expression of p21 renders lymphocytes less responsive to allo-and mitogen-induced proliferation and prolongs allograft survival in a rat heart transplant mode[4, 5]. Analysis of the consequences of CD4+CD25+ T cell and p21 deficiency demonstrates a close similarity regarding

increased immune activation; break in tolerance and development of lupus occurs[9, 10]. Therefore, p21 may play a crucial role in the generation of T regulatory cells. We exploited this strong association between FoxP3 expression and regulatory function in p21 deficient lymphocytes and CD4+CD25- T cells. .It is believed that $CD4^{+}CD25^{+}$ regulatory T cells [Treg's] play a key role in inducing tolerance to allogeneic organ transplants. TGF-β plays a pivotal role in suppression of lymphocyte proliferation by Treg's and effectively diminishes inflammation and negatively regulates immune functions by inducing cell anergy[9]. TGF-β also induces the expression of Foxp3, a molecule associated with the development of Treg's[11]. Though numbers of such studies have highlighted the role of TGF-β in the generation and maintenance of Treg's, this does not seem to be universally true. CD4+CD25+ regulatory T cells developed in complete absence of endogenous TGF-β expression, and that autocrine TGF-β expression was not necessary for these cells to suppress inflammation *in vivo*[12-14] as the neutralization of TGF-β with either monoclonal antibody [mAb] or soluble TGF-β RII-Fc failed to reverse *in vitro* suppression mediated by resting or activated CD4+ CD25+ T cells. Responder T cells from Smad3 $^{-/-}$ or dominant-negative TGF-β type RII transgenic [DNRIITg] mice, both unresponsive to TGF-β-induced growth arrest, were as susceptible to CD4+CD25+-mediated suppression as T cells from wild-type mice. Furthermore, CD4+CD25+ T cells from neonatal TGF-$\beta^{-/-}$ mice were functionally not different from TGF-beta1$^{+/+}$ mice[15,16]. Therefore, CD4+CD25+ suppressor function can occur independently of TGF-β, . Since TGF-β's anti-proliferative effects are mediated by p21, we speculated that these effects of TGF-β may be via induction of p21.

Other studies also indicate similarities between cells deficient in p21 and CD4+CD25+ population, the deficiency of p21 and CD4+CD25+ cells[17-23] results in the development of spontaneous lupus-like disease. The deficiency of p21 resulted in immune activation, homeostatic proliferation and manifestation of lupus-like disease. These findings were supported by studies[17-18], which demonstrated that the $p21^{-/-}$ mice have increased lymphocyte activation, antibodies against dsDNA, lymphoadenopathy and glomerulonephritis similar to lupus patients. A mimic of p21 that negatively regulated T cell proliferation exhibited protective effects in a mouse model of lupus[24]. TGF-β's anti proliferative effects are mediated by cyclin inhibitor p21 and our studies have demonstrated that CsA and tacrolimus induce p21's gene and protein expression, which is dependent on TGF-β expression[7, 25]. We have reported[4, 5] that *in vitro* and *in vivo* overexpression of p21 decreased response of mitogens on lymphocyte proliferation and increased responsiveness to the antiproliferative effects of CsA. Therefore, it is plausible that p21 plays a significant role in the generation of T regulatory cells.

Apoptosis is one of the critical events in maintaining immune tolerance to self and non-self antigens. It is likely that inhibition of apoptosis in lymphocytes leads to break in tolerance, alloimmune activation and inflammation[26]. This is supported by the role of dendritic cells [DC] in tolerance[27]. Induction of apoptosis in T cells treated with co-stimulatory block and sirolimus paralleled with tolerance[28, 29], the mechanism of this is not clear but lymphocyte undergoing apoptosis have been shown to secrete anti-inflammatory molecules such as IL-10 and TGF-beta[30] known to be the inducers of T regulatory cells. Interestingly, this role of apoptosis in induction of tolerance fits well in support of the role p21 in this process. The role of p21 in induction of apoptosis in cancer

cells[31] and T lymphocytes[32] has been related to its anti-proliferative effects. Furthermore, in p21 transgene mice restricted to the T cell lineage, T cells were more responsive to cell death confirming the proapoptotic effect of p21[33]. Based on these results p21 seems to mediate generation of T Regs and immune tolerance.

Interestingly, our interesting findings can also be explained with number of factors such as therapeutic agents and transcription factors such as HDAC inhibitors, RUNX transcription factors, and nuclear factor of activated T cells [NFAT] that interact with both p21 and FoxP3. HDAC inhibitors [HDACi] have been shown to increase Foxp3 expression through enhancing the numbers and functions of $Foxp3^+$ $CD4^+CD25^+$ T regulatory cells, suggesting the possibility of increasing the suppressive properties of T regulatory cells using pharmacologically agents[34]. Interestingly, several studies have shown that HDAC inhibitors strongly activate the expression of p21[35-37]. Thus, our studies show important new mechanisms by which HDACis can modulate inflammatory and immune responses *in vivo* through the induction of T regulatory cells possibly mediated by the induction of p21. Since it is known that the inhibition of HDAC *in vitro* and *in vivo* increases the numbers of $Foxp3^+$ T cells and there is a direct relationship between the presence of p21 and FoxP3+ cells, it is again suggesting that p21 regulates the function of T regulatory cells. Other studies[38] have observed the histone deacetylase [HDAC] inhibitors [phenylbutyrate and trichostatin A] by hyperacetylation of histone not only induced the expression of p21 in synovial cells but also inhibited the expression of tumor necrosis factor-alpha in affected tissues in adjuvant arthritis, an animal model of RA. Nuclear factor of activated cells [NFAT] also interacts with Foxp3 and Runx1[39], which involved TIP60 to facilitate FoxP3 complex-mediated repression of IL-2

production. TIP60 is known to induce expression of p21 asTip60 knockdown inhibited induction of p21 and its over-expression resulted in increase p21 expression levels in 293T cells[40-41]. NFAT components, NFATc and NFATp with opposite functions also regulate expression of FoxP3 negatively and positively, respectively. Our studies have shown that the suppression of T cell proliferation by recombinant p21 protein results in an increased NFATp and decreased NFATc expression that correlated with IL-2 expression[6]. These results suggest that p21 may mediate the suppression of T lymphocyte proliferation through FoxP3 positive T regulatory cells. Similarly, RUNX Runx1 [Runt-related transcription factors], which is crucially required for normal hematopoiesis including thymic T-cell development also participate in generation of FoxP3 cells, suppression or activation of cellular proliferation and the actions include the relevant activity of both FoxP3 and p21[42-44]. Members of RUNX family RUNX 1, 2 and 3 have distinctive functions. Additionally, Foxp3 also interacts with two other members of the AML/Runx protein family, AML2 [Runx3] and AML3 [Runx2], albeit in different ways, its interaction with RUNX3 is positive and RUNX2 is negative on cellular proliferation including hematopoietic, immune and cancer cells. The effect on immune system and interaction between RUNX family members is reflected by their stimulatory and suppressive effects on p21. More recently[45], p21 was shown to be a downstream target for FoxP3, because it served as an important target for all major tumor suppressor genes and the authors speculated the possibility of reactivation of p21 in cancer by inducing FoxP3. These results are supportive of our findings suggesting that the activation of FoxP3 by p21 may prove to be a more relevant to the development of immune tolerance.

The results therefore, signify the possible therapeutic potential of p21 in induction/generation of T regulatory cells in the recipients providing a milieu for immune tolerance in organ transplantation.

Acknowledgement: The author is thankful to Matthew Plummer for his excellent technical assistance. There are no funding sources and disclosures relevant to this work.

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Figure Legends:

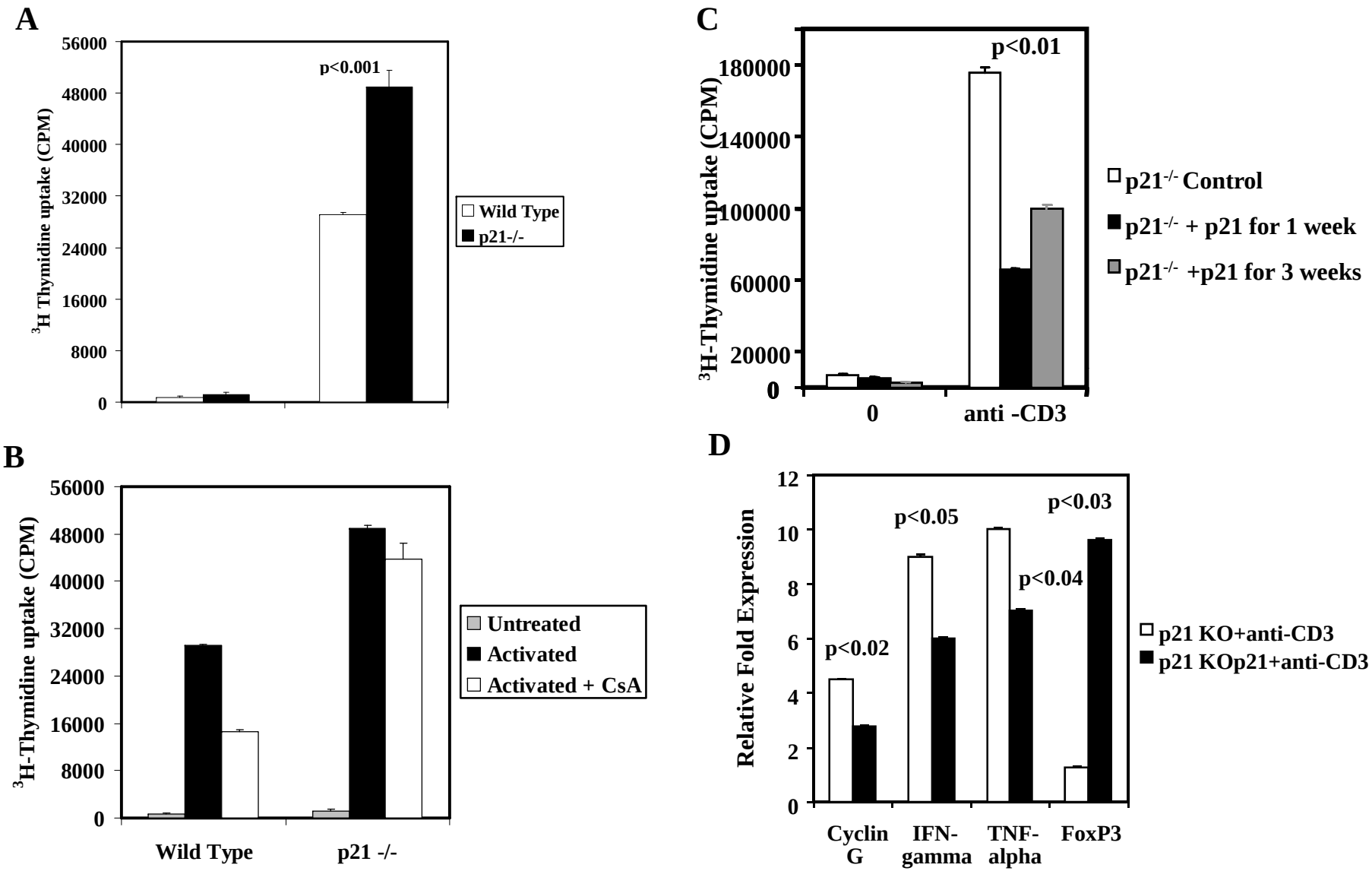
Figure 1: Effect of cyclin kinase inhibitor p21 modulation on lymphocyte proliferation and inflammation. **A:** A statistical significant [$p<0.001$] increased proliferation of splenocytes from $p21^{-/-}$ [closed bars] compared to WT mice [open bars] is shown. **B:** Inhibition of proliferation of activated splenocytes was observed only in WT and not $p21^{-/-}$ mice. **C:** Statistically significant [$p<0.01$] decreased proliferation in p21 gene restored $p21^{-/-}$ mice is shown after one [closed bars] and three [gray bars] is shown. **D**: Overexpression of p21 significantly decreased cyclin G [$p<0.02$], IFN-gamma [$p<0.05$], TNF-alpha [$p<0.04$] and increased FoxP3 [$p<0.03$] mRNA expression

Figure 2: Quantification of CD4+CD25+ T cells and FoxP3 mRNA expression in WT and p21-/- mice. A: Significantly [$p<0.002$] lower number of CD4+CD25+ T cells is shown in $p21^{-/-}$ compared to WT mice. **B:** Expression of FoxP3 mRNA is seen only in splenocytes from WT but not $p21^{-/-}$ mice. Similar expression of house-keeping gene □-actin is shown. **C:** Amplification for FoxP3 mRNA was seen only with CD4+CD25+ T cells, analysis of mRNA expression normalized to β-actin is shown in **D**, and E: Mean ± SEM of normalized fold expression of FoxP3 mRNA in CD4+CD25+, CD4+CD25- and mixture of CD4+CD25+ T cells from both mice is shown in **E**.

Figure 3: Differential response of CD4+CD25+ and CD4+CD25- T cells to mitogenic stimuli: **A**: CD4+CD25- T cells proliferated significantly higher [$p<0.001$] compared to CD4+CD25+ cells in response to anti-CD3 antibody. **B**: Proliferation of CD4+CD25+ not CD4+CD25- T cells was inhibited by CsA. CD4+CD25+ T cells [**C**] not CD4+25-activated T cells induced TGF-β and p21 mRNA [**D**] in response to CsA.

Figure 4: Similarities between proliferation responses of CD4+CD25+, CD4+CD25-, and immune cells from WT and p21$^{-/-}$ mice: **A:** CD4+CD25+, CD4+CD25- or a mixture [1:1] of CD4+CD25+/- T cells were activated with anti-CD3 antibody, Cd4+CD25- T cells proliferated higher than CD4+CD25+ cells and the proliferation of the mixture of CD4+CD25+/- T cells was almost in the median range. B: mRNA expression of T regulatory specific markers FoxP3, ILT3 and GITR reflects the differential proliferation of CD4+CD25+, CD4+CD25- and mixture of these cells. **C**: Results from autologous MLR assay using splenocytes from wild type and p21$^{-/-}$ mice as responder and stimulators, Gray Bars; WT stimulators and responders, Closed Bars; p21$^{-/-}$ stimulator and responder; Open Bars: WT stimulator+ p21$^{-/-}$ responders. WT stimulator splenocytes decreased proliferation of p21$^{-/-}$ splenocytes. **D**: FoxP3 mRNA expression correlates with inhibitory effects of WT splenocytes on p21$^{-/-}$ splenocytes in an autologous MLR assay.

Figure 1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Wild Type
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p<0.01
p21$^{-/-}$ Control
p21$^{-/-}$ + p21 for 1 week
p21$^{-/-}$ +p21 for 3 weeks
0
anti -CD3
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12
10
8
6
4
2
0
Relative Fold Expression
p<0.02
p<0.05
p<0.04
p<0.03
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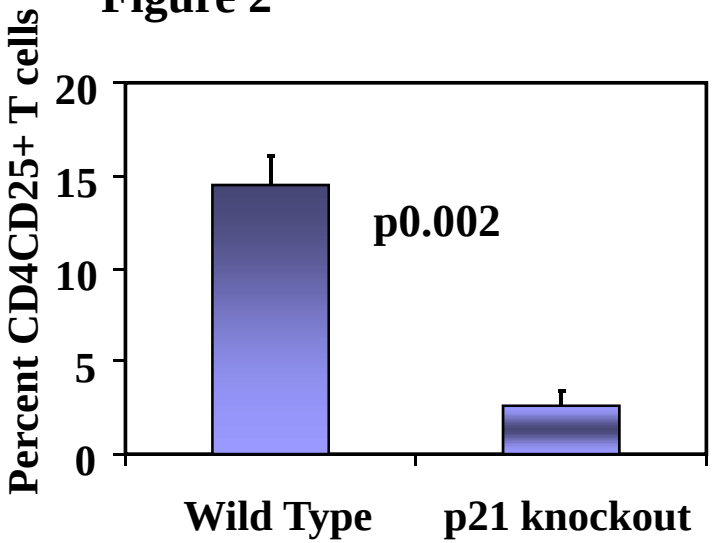
Cyclin G
IFN-gamma
TNF-alpha
FoxP3

Figure 2

A



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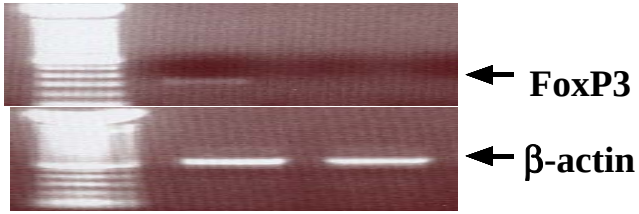
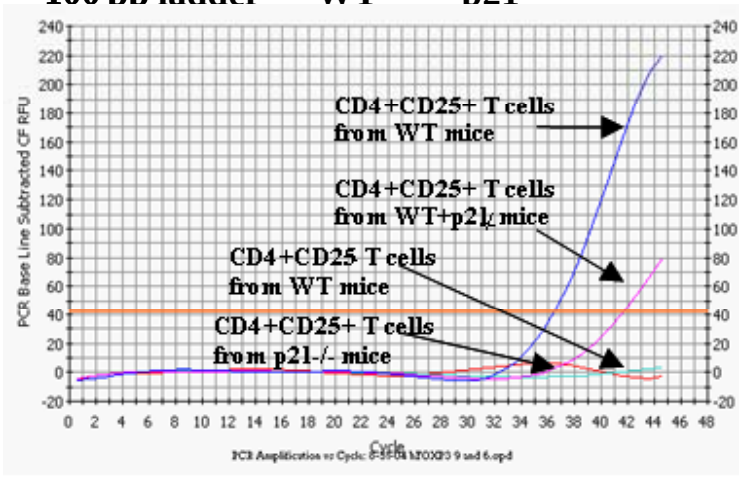
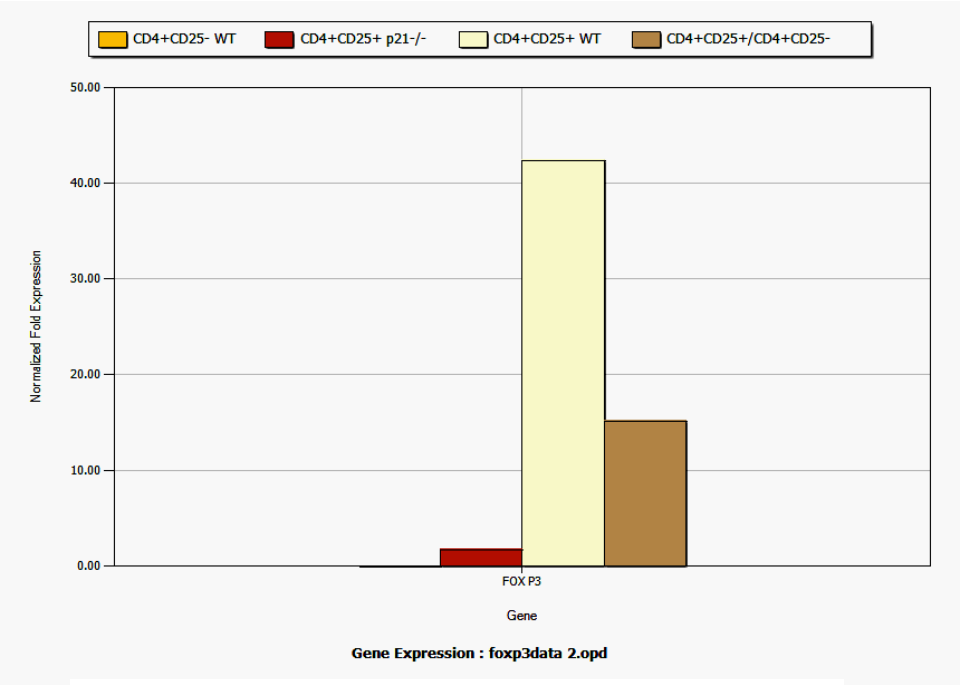


C



D



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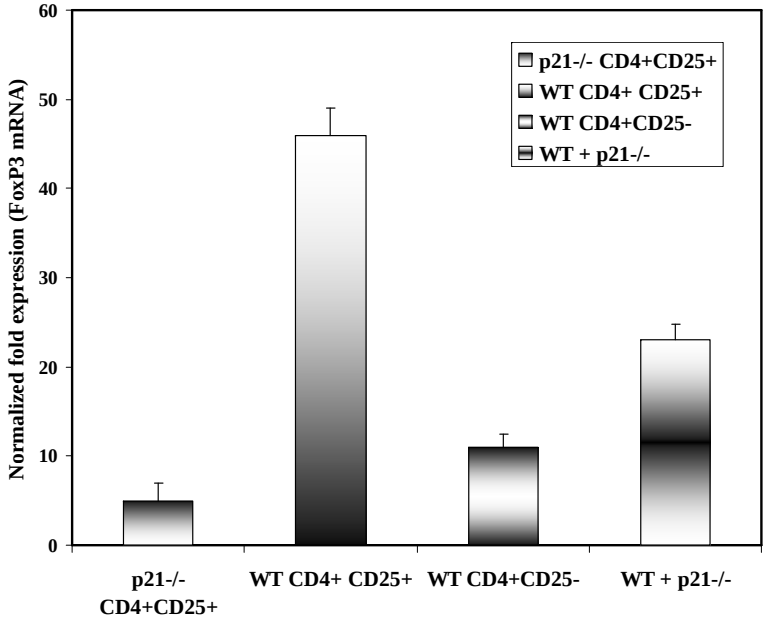


Figure 3

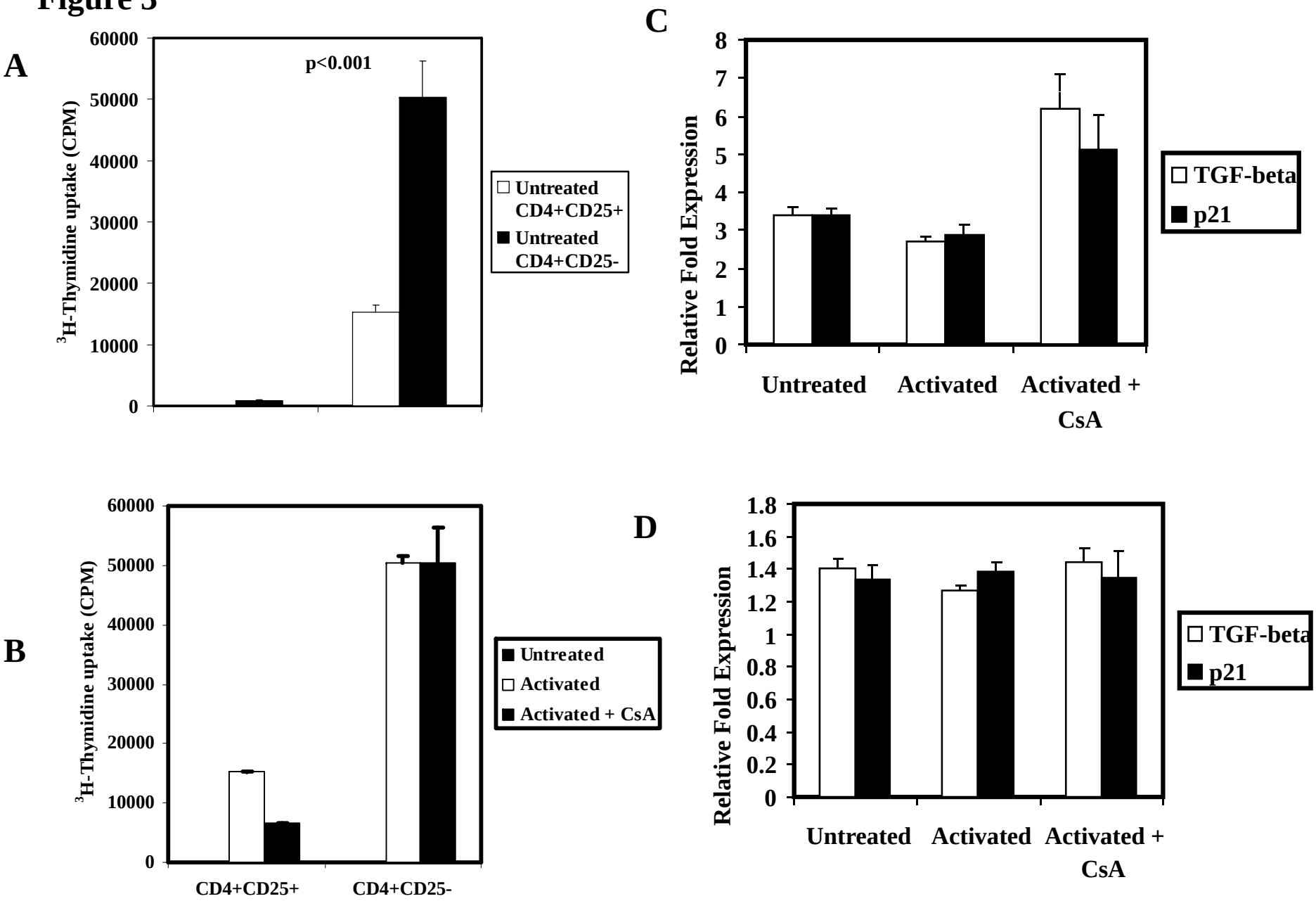
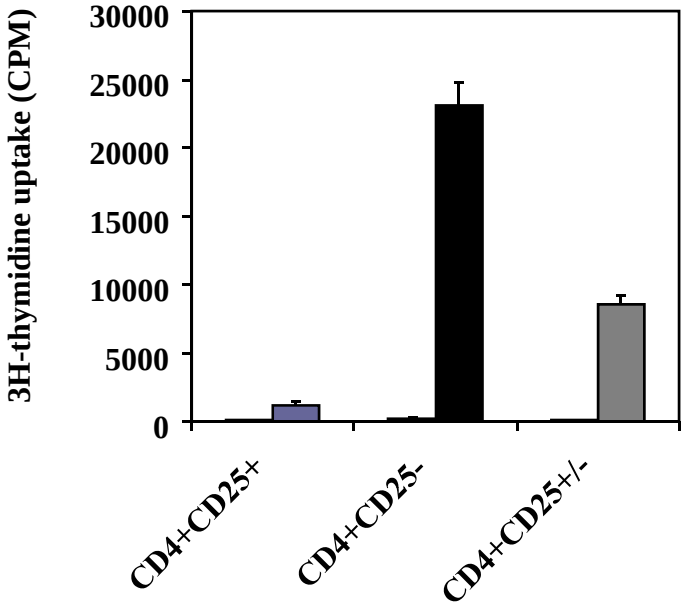
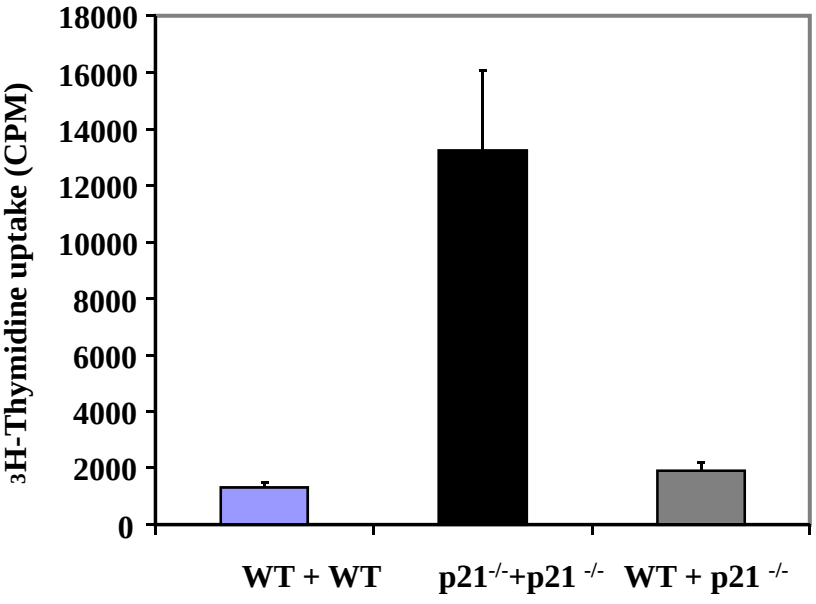

A
p<0.001
^{3}H-Thymidine uptake (CPM)
60000
50000
40000
30000
20000
10000
0
Untreated CD4+CD25+
Untreated CD4+CD25-
B
^{3}H-Thymidine uptake (CPM)
60000
50000
40000
30000
20000
10000
0
CD4+CD25+
CD4+CD25-
Untreated
Activated
Activated + CsA
C
Relative Fold Expression
8
7
6
5
4
3
2
1
0
Untreated
Activated
Activated + CsA
TGF-beta
p21
D
Relative Fold Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
Activated
Activated + CsA
TGF-beta
p21

Figure 4

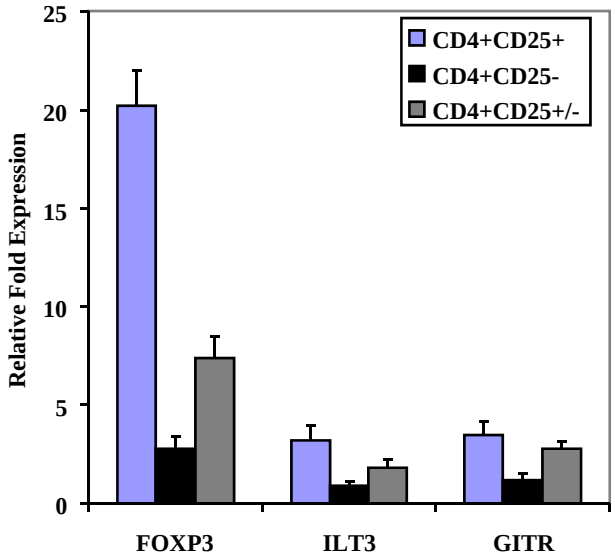
A



C



B



D