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# Selective expression rather than specific function of Txk and Itk regulate $T_H1$ and $T_H2$ responses[§]

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## Abstract

Itk and Txk/Rlk are Tec family kinases expressed in T cells. Itk is expressed in both $T_H1$ and $T_H2$ cells. By contrast, Txk is preferentially expressed in $T_H1$ cells. Although Itk is required for $T_H2$ responses in vivo and Txk is suggested to regulate IFNγ expression and $T_H1$ responses, it remains unclear whether these kinases have distinct roles in $T_H$ cell differentiation/function. We demonstrate here that Txk null $CD4^+$ T cells are capable of producing both $T_H1$ and $T_H2$ cytokines similar to those produced by WT $CD4^+$ T cells. To further examine whether Itk and Txk play distinct roles in $T_H$ cell differentiation and function we examined Itk-null mice carrying a transgene that expresses Txk at levels similar to the expression of Itk in $T_H2$ cells. Using two $T_H2$ model systems: allergic asthma and Schistosome egg-induced lung granulomas, we found that the Txk transgene rescued $T_H2$ cytokine production and all $T_H2$ symptoms without notable enhancement of IFNγ expression. These results suggest that Txk is not a specific regulator of $T_H1$ responses. Importantly, they suggest that Itk and Txk exert their effects on $T_H$ cell differentiation/function at the level of expression.

## Introduction

Txk/Rlk (hereafter referred to as Txk) and Itk are distantly related members of the Tec family of tyrosine kinases that are involved in signaling downstream from the TCR. While Txk has a palmitoylation site instead of a PH domain that allows it to be constitutively associated with lipid raft membrane fractions, Itk requires the activation of PI3 kinase for recruitment to the membrane via its PH domain (1,2). Mutations affecting Itk in mice lead to altered T cell

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development and mature T cell function with reduced TCR-induced proliferation and impaired IL-2 production in vitro ((3-6) see (7) for review)). One of the most dramatic phenotype of *Itk*$^{-/-}$ mice is their defect in $T_H2$ responses in vivo. *Itk*$^{-/-}$ mice are incapable of developing allergic asthma and have decreased responses to challenge with a number of $T_H2$-inducing parasites including the eggs of *Schistosome mansoni* or the worm *Nippostrongyloides brasilienses* (8-11). Indeed, in some cases, Itk-deficient mice have been found to mount $T_H1$ responses to $T_H2$-inducing pathogens. In contrast, overexpression of Txk has been associated with increased expression of IFN-γ, a $T_H1$ cytokine (12-15). Txk has been found to bind directly to a sequence in the IFN-γ promoter, suggesting a direct role for Txk in driving IFN-γ transcription and $T_H1$ responses (16). Together, these data suggested that Itk and Txk have distinct roles in $T_H2$ and $T_H1$ differentiation or function respectively.

Nonetheless, the exact mechanism by which the Tec kinases influence T helper cell differentiation remains controversial. While some data suggest that Itk induces $T_H2$ differentiation by suppressing the expression of T-bet (17), other reports propose that Itk but not Txk directly interacts with and tyrosine phosphorylates T-bet, promoting its interaction with GATA3, which suppresses the latter's activity (18). It has also been proposed that Itk may modulate $T_H2$ differentiation by virtue of its expression: Itk is expressed at higher levels than Txk in naïve T cells and while both Itk and Txk are expressed in $T_H1$ cells, Txk is downregulated in $T_H2$ cells, leaving Itk as the major Tec kinase (17). Consistent with this idea, recent data argue that Itk is not required for $T_H2$ differentiation per se, but rather is required for effector function of differentiated $T_H2$ cells (19). These data suggest it may not be the intrinsic function of these kinases, but rather their patterns of expression that determines their roles in T helper cell differentiation and function.

To evaluate these questions, we examined whether forced over-expression of Txk in T cells could rescue $T_H2$ responses in *Itk*$^{-/-}$ mice using transgenic mice that drive expression of Txk at levels similar to Itk in $T_H2$ cells. If these kinases have distinct $T_H1$ and $T_H2$-inducing properties, one would predict that overexpression of Txk would preferentially drive $T_H1$ responses and $T_H2$ defects in *Itk*$^{-/-}$ mice may be exacerbated. However, if defects in $T_H2$ responses in *Itk*$^{-/-}$ mice are secondary to the low levels of expression of Tec kinases in $T_H2$ cells in the absence of Itk, expression of the Txk transgene may rescue these responses. Utilizing two systems: 1) a murine model of allergic asthma and 2) challenge with the eggs of *Schistosome mansoni*, a strong $T_H2$ inducing parasite (8,10), we demonstrate that *Itk*$^{-/-}$ mice expressing a Txk transgene can rescue $T_H2$ responses, with no evidence of overexpression of $T_H1$ cytokines. Our results thus strongly suggest that the effects of Itk and Txk on T helper cell function may result from the differential patterns of expression of these kinases.

## Materials and Methods

### Mice

Wild-type (WT), *Txk*$^{-/-}$ (20) *Itk*$^{-/-}$ (6) and *Tg(CD2-Txk)* (13), were used between 6-12 week of age and were backcrossed on the C57BL/6 background for at least 5 generations. *Tg(CD2-Txk)Itk*$^{-/-}$ mice were generated by breeding *Itk*$^{-/-}$ and *Tg(CD2-Txk)* mice. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Pennsylvania State University and the NIH.

### Flow cytometry

Single cell preparations were made from thymi and spleens. Red blood cells were lysed from spleens using ammonium chloride lysis solution. Cells were preincubated with anti-CD16, then stained with combinations of: anti-CD8α (Clone 53-6.7, FITC or PE), CD62L (MEL-14, PE),

CD4 (RM4-5, PerCP-Cy5.5), CD44 (IM7, APC), CD24 (M1/69, FITC) (eBioscience, San Diego, CA or BD Biosciences/Pharmingen, San Jose, CA).

### Allergic asthma induction

Mice were sensitized with ovalbumin (Sigma-Aldrich) complexed to aluminum hydroxide (10 μg ovalbumin/1 mg alum; Pierce) intraperitoneally in a total volume of 200 μl on days 0 and 5. Mice were later challenged intranasally with ovalbumin from days 12 through 15 (at a concentration of 2 mg/ml, for a total of 40 μg total exposure). Development of allergic asthma was measured by analyzing AHR on day 16 using a custom made mechanical ventilator as previously described (9,10,21). Mice were then sacrificed, BALF obtained and lungs sectioned and stained using H&E or PAS as detailed (9,10).

### Schistosome egg injection and analysis of response

Mice were primed and challenged with *S. mansoni* eggs as described using frozen eggs for primary injections and fresh eggs for secondary injections 10 days later (8). Nine to 10 days after secondary challenge, lungs were collected and processed as described for analysis of granulomas (8).

### Quantitative Real-time RT-PCR analysis

After analysis of AHR, RNA was extracted from the lungs of mice using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). cDNA was generated with a kit from Amersham Biosciences (Piscataway, NJ). Quantitative RT-PCR was then performed for IL-4, IL-13, IFN-γ, CCL-7 and CCL-11, with GAPDH used as a housekeeping gene and the data expressed as $2^{-\Delta\Delta CT}$ (9). To quantify the expression level of Itk and Txk, Quantitative RT-PCR was performed on cDNA from native $CD4^+$ T cells or $CD4^+$ T cells differentiated to $T_H2$ cells. Signals were compared to standards generated from plasmids carrying the cDNA for Itk and Txk.

### Analysis of calcium responses

$CD4^+$ T cells were purified from spleens by negative selection using MACS mouse $CD4^+$ T cell isolation kit microbeads (Miltenyi, Auburn, CA), with the addition anti-NK1.1(BD Biosciences/Pharmingen, San Jose, CA). Cells were labeled with 5 μg/ml Fluo3 and 5 μg/ml Fura-red (Molecular Probes/Invitrogen, Carlsbad, CA). Cells were kept at room temperature, then stimulated at 37°C with 15 μg/ml biotinylated anti-CD3 (2C11) with the addition of 8 μg/ml streptavidin. Data were collected on a FacsCalibur for 10 minutes and analyzed using FlowJo software (TreeStar, Ashland, OR).

### Analysis of lymphocyte proliferation and cytokine secretion in response to antigen challenge

Purified splenocytes obtained from ovalbumin challenged mice were cultured with 10 and 100 μg/ml of OVA ($2 \times 10^5$ cells/well). After 72 hours of culture, cells were pulsed with [$^3$H]-thymidine for 18 h and incorporated radioactivity determined. Cytokine secretion was analyzed by stimulating splenocytes at $2 \times 10^6$/ml with 100 μg/ml OVA for 96 h following which supernatants were harvested. Cytokine concentration in supernatants and BAL were determined using a Luminex system (Biorad, Austin, TX), with plates from Lincoplex (Millipore Systems, Billerica, MA). In mice exposed to *S. mansoni* eggs, cells from draining mediastinal lymph nodes and spleens were collected and restimulated with SEA as described (8). Three days later, cells were stimulated with PMA and Ionomycin in the presence of Golgi-stop (BD Biosciences/Pharmingen, San Jose, CA), stained for CD4 and intracellular IL-4, -5, -10 and IFN-γ using specific antibodies and analyzed by flow cytometry.

### In vitro differentiation to $T_H1$ and $T_H2$ cells

T cells were purified by T cell isolation columns (R&D, Minneapolis, MN) and then sorted for $CD4^+CD62L^{hi}$ and $CD44^{lo}$ naïve cells. Cells were stimulated with 1 μg/ml anti-CD3 plus anti-CD28 in the presence of mitomycin-treated T-depleted splenocytes for 2-3 days, then restimulated with PMA and Ionomycin in the presence of golgi-plug, stained for intracellular levels of IFN-γ and IL-4 and analyzed by flow cytometry as above. $T_H0$ conditions contained no extra cytokines, $T_H1$ conditions included 40 ng/ml IL-12 and 10 μg/ml anti-IL-4; $T_H2$ conditions included 40 ng/ml IL-4 and 10 μg/ml anti-IL-12 and anti-IFN-γ. Cytokines were obtained from Peprotech (Rocky Hill, NJ).

### Data analysis

Statistical evaluation was conducted by using the Student's *t* test with a probability value of $p<0.05$ considered statistically significant.

## Results

### Txk null T cells are not defective in $T_H1$ or $T_H2$ cytokine secretion

Txk has been suggested to specifically regulate the production of IFN-γ in $T_H1$ cells. To determine if T cells lacking Txk have defects in this function, we analyzed T cells from Txk null mice (20). We first confirmed that Txk null mice had similar T cell subpopulations to WT mice (Fig. 1a (20)). Consistent with these observations, $Txk^{-/-}$ $CD4^+$ T cells showed normal TCR-induced $Ca^{2+}$ mobilization (Fig. 1b), supporting the conclusion that at this level of analysis, T cells from Txk null mice exhibit no significant abnormalities. We next analyzed naïve $CD4^+$ T cells that were differentiated under $T_H1$ or $T_H2$ conditions (Fig. 1c). Txk null T cells were able express both $T_H1$ and $T_H2$ cytokines similar to WT T cells. Thus, although it has been proposed that Txk is a $T_H1$-inducing kinase, these data support the conclusion that Txk is not required for the generation of IFN-γ in $T_H1$ cells.

### The CD2 promoter driven Txk transgene is expressed in $T_H2$ cells at similar levels to endogenous Itk

Although the above results suggest that Txk is not required for IFN-γ expression, Txk is expressed at lower levels than Itk (Fig. 1d), and may have unique functions that may not be obvious due to its low level of expression. To evaluate whether Txk can compensate for Itk if expressed at higher levels, we utilized a transgenic mouse model in which Txk was overexpressed using the CD2 promoter (*Tg(CD2-Txk)*). When crossed onto an Itk null background (referred to here as *Tg(CD2-Txk)*$Itk^{-/-}$), expression of Txk was able to rescue the known calcium signaling defect downstream of TCR stimulation that occurs in T cells lacking Itk (Fig. 1b), similar to what has been reported in the thymus (13). However, although expression of this Txk transgene has been shown to improve positive selection in Itk-deficient mice, *Tg(CD2-Txk)*$Itk^{-/-}$ mice still had low numbers of peripheral T cells, even lower than Itk-deficient mice (Supplemental Table 1).

To further evaluate the Txk transgene, we used qRT-PCR to evaluate the relative expression levels of *Txk* and *Itk* mRNA in freshly isolated $CD4^+$ T cells and in cells that were differentiated under $T_H2$-inducing conditions. Although the expression of *Txk* in freshly isolated $CD4^+$ T cells was higher (17-fold) than endogenous *Txk* in WT mice, it was only 5-fold higher than the level of expression of Itk mRNA as measured by qRT-PCR (Fig. 1d). As previously reported, endogenous Txk expression in WT $T_H2$ cells was dramatically reduced in $T_H2$ cells (17). However, in $T_H2$ cells from *Tg(CD2-Txk)*$Itk^{-/-}$ mice, *Txk* expression remained high, similar to levels of Itk in WT $T_H2$ cells (Fig. 1d). Thus, in $T_H2$ cells Txk expression was similar to Itk

expression on a copy number basis. We therefore utilized this transgene on the *Itk*$^{-/-}$ background to evaluate the ability of *Txk* to complement Itk function in $T_H2$ cells.

## Rescue of allergic airway inflammation and airway hyperresponsiveness (AHR) in mice lacking Itk by expression of Txk transgene

To determine the effect of expression of *Txk* in Th2 cells, we analyzed the development of allergic asthma, a disease dependent on $T_H2$ cells and cytokines. OVA immunized and challenged WT and *Itk*$^{-/-}$ mice, as well as mice expressing the *Txk* transgene on an *Itk*$^{-/-}$ background (*Tg(CD2-Txk)Itk*$^{-/-}$) were evaluated for airway resistance in response to methacholine challenge as a measure of AHR. In these experiments, while WT mice developed significant airway resistance, *Itk*$^{-/-}$ mice responded poorly as previously reported (Fig. 2a) (9). However, *Tg(CD2-Txk)Itk*$^{-/-}$ transgenic mice exhibited significant levels of airway resistance, similar to the WT mice (Fig. 2a).

We next analyzed airway inflammation and mucous production, factors that can contribute to the development of allergic asthma in this model. Histological evaluation of lung sections revealed that *Tg(CD2-Txk)Itk*$^{-/-}$ mice exhibit massive leukocyte infiltration in the lung, which was similar or higher than that observed in WT mice (Fig. 2b). In contrast, *Itk*$^{-/-}$ mice showed reduced leukocyte infiltration, as previously reported (9,10). Increased thickening of the epithelial cell lining of the bronchioles and mucous production by airway goblet cells was also observed in *Tg(CD2-Txk)Itk*$^{-/-}$ mice, similar to that seen in WT mice (Fig. 2b).

## Expression of Txk transgene in Itk-null mice enhances production of $T_H2$ cytokines in response to allergic inflammation

$T_H2$ specific cytokines such as IL-4, IL-5 and IL-13 are involved in inducing allergic airway inflammation (22,23). To examine whether expression of *Txk* transgene rescued production of $T_H2$ cytokines, we first analyzed cytokine production from splenic T cells of mice immunized and challenged with OVA. Stimulation with OVA in vitro induced proliferation of T cells from WT, *Tg(CD2-Txk)Itk*$^{-/-}$ and *Itk*$^{-/-}$ OVA-challenged mice, although those from *Itk*$^{-/-}$ mice exhibited reduced proliferation in comparison to WT mice (Fig. 3a) (10). Splenocytes from *Tg(CD2-Txk)Itk*$^{-/-}$ mice, however, had proliferative responses equivalent to WT mice, and the *Txk* transgene rescued IL-4, IL-5 and IL-13 secretion from these cells in vitro (Fig. 3b). Since *Txk* is suggested to regulate the expression of $T_H1$ cytokines such as IFN-γ, we also examined the expression of IFN-γ. Strikingly, *Tg(CD2-Txk)Itk*$^{-/-}$ mice did not secrete elevated levels of IFN-γ as would be expected if it specifically regulated IFNγ (Fig. 3b).

To further examine the expression level of $T_H2$ cytokines in the lungs upon induction of allergic asthma, we measured the expression level of IL-4 and IL-13 mRNA in the lungs of OVA immunized and challenged WT, *Itk*$^{-/-}$ and *Tg(CD2-Txk)Itk*$^{-/-}$ mice using real-time quantitative RT-PCR. As shown in Figure 3c, there was enhanced expression of IL-4 and IL-13 mRNA in the *Tg(CD2-Txk)Itk*$^{-/-}$ mice in comparison to WT and *Itk*$^{-/-}$ mice. By contrast, the mRNA levels of IFN-γ were similar in all three strains of mice (Fig. 3c). These findings were further confirmed by the analysis of BAL fluid for the level of $T_H2$ cytokines in these mice, indicating that expression of *Txk* rescued the $T_H2$ mediated inflammation in the lungs of OVA challenged mice (Fig. 3d). Therefore the Txk transgene does not appear to lead to enhanced $T_H1$ differentiation or IFN-γ production, nor does it block $T_H2$ differentiation as would be observed if Txk were a $T_H1$-inducing kinase (12-16). Higher levels of IL-13 mRNA but not protein were also observed in the *Tg(CD2-Txk)Itk*$^{-/-}$ mice in comparison to WT. These results could be due to IL-13 mRNA expression in other inflammatory cells such as eosinophils and mast cells, recruited to the lungs in response to the disease. Nonetheless, we did not observe higher levels of the IL-13 protein, suggesting this message may not be fully translated in these

cells. Altogether, these data indicate that in the absence of Itk, *Txk* expression can lead to the generation of a $T_H2$ response both systemically and in the lungs.

### Rescue of CD4$^+$ T cell recruitment in Itk-null mice expressing Txk transgene

Analysis of CD4$^+$ T cell numbers in the lungs revealed that while WT mice could recruit these cells into the lung during airway inflammation, as previously reported, Itk-null mice could not (10,24). However, *Tg(CD2-Txk)Itk*$^{-/-}$ mice had similar numbers of CD4$^+$ T cells in the lungs compared to WT mice, indicating that expression of *Txk* was able to rescue migration and recruitment of T cells into the lung (Fig. 4). These results were not secondary to increased numbers of CD4$^+$ T cells in *Tg(CD2-Txk)Itk*$^{-/-}$ mice as these mice have even lower numbers of mature CD4$^+$ T cells as *Itk*$^{-/-}$ mice (Supplemental Table 1). These data thus confirm that in vivo, *Txk* can rescue specific functions of Itk that lead to the recruitment of leukocytes into the lungs during the development of allergic asthma.

### The Txk transgene rescues $T_H2$ responses to Schistosome mansoni eggs

To examine induction of $T_H2$-mediated responses using a different model, we analyzed responses to i.v. injection of *S. mansoni* eggs. Injection of *S. mansoni* eggs i.v. into mice results in the formation of eosinophilic granulomas in the lung in a $T_H2$ cell dependent manner. In *Itk*$^{-/-}$ mice, the size of these granulomas is reduced (Fig. 5a) (8). However, consistent with our observations in the OVA induced model, injection of *S. mansoni* eggs into *Tg(CD2-Txk) Itk*$^{-/-}$ mice resulted in granulomas of similar size to those seen in WT mice (Fig. 5a). In contrast, overexpression of *Txk* on a WT background also did not increase granuloma size (Fig. 5a). To evaluate $T_H2$ cytokine production in this model, we examined cytokine production by intracellular staining of cells from the spleen and draining mediastinal nodes of mice injected with *S. mansoni* eggs. Analyses of splenic and lymph node T cells restimulated in vitro with Schistosome egg antigen (SEA) confirmed that Itk-null mice had fewer cells producing the $T_H2$ cytokines IL-4, -5 and -10. However, *Itk*$^{-/-}$ mice carrying the *Txk* transgene had similar numbers of cells making these cytokines to that seen in WT mice, confirming that $T_H2$ differentiation was rescued. Again, *Tg(CD2-Txk)Itk*$^{-/-}$ mice did not show elevated numbers of IFN-γ producing cells (Fig. 5b,c). These mice also did not show increased percentages of cells expressing $T_H2$ cytokines compared to WT cells, demonstrating that the cells were not merely over-responsive due to the expression of the *Txk* transgene. Similarly, overexpression of *Txk* on a WT background did not increase the level of $T_H1$ or $T_H2$ cytokines (Fig. 5b,c), again arguing that expression of the Txk transgene did not merely result in increased activation of these cells. These data are in keeping with the finding that T cells from these mice do not exhibit higher levels of calcium influx in response to TCR stimulation (see Fig. 1). Thus, expression of *Txk* transgene can rescue patterns of $T_H2$ cytokine production in response to multiple $T_H2$ inducing agents.

## Discussion

The Tec kinases Itk and Txk are both expressed in T cells and regulate their development, activation and function (7). A role for Itk has been demonstrated for the production of Th2 cytokines, while roles for Txk are less clear (8-11,17,19). Prior work suggests that Txk regulates IFN-γ production and thus $T_H1$ development, however a specific role of Txk in the regulation of $T_H1$ specific cytokine production is still unresolved (12-16). Our data argue that Txk does not specifically drive expression of this $T_H1$ cytokine. First, Txk null T cells do not exhibit defects in producing IFN-γ, arguing that TXK is not essential for IFN-γ production. More importantly, in the models examined here, overexpression of Txk did not induce elevated levels of IFN-γ and prevent the development of $T_H2$ cell mediated disease. Instead, overexpression of Txk rescued production of $T_H2$ type cytokines including IL-4, -5 and 13 in the lungs of *Tg(CD2-Txk)Itk*$^{-/-}$ mice in response to $T_H2$-inducing agents. It should be noted

that these observations do not rule out the possibility that Txk could indirectly regulate IFN-γ production via interaction with other factors that are specifically expressed in $T_H1$ cells.

Txk is the most distantly related family member of the Tec family of tyrosine kinases. This protein has a N-terminal palmitoylation site instead of a PH domain found in Itk and other Tec kinases, allowing it to be anchored constitutively in the plasma membrane. Thus Txk, unlike Itk, does not require the activation of PI3 kinase in order to be recruited to the plasma membrane (1,2). This would suggest specific and unique functions for Txk. However, we show that when expressed at similar levels to Itk, Txk can functionally replace Itk for the induction of predominant $T_H2$ responses in vivo by enhancing the expression of $T_H2$ specific cytokines. Our data provide strong evidence that Txk can function to rescue $T_H2$ responses in the absence of Itk, including AHR, airway inflammation, granuloma formation, T cell recruitment and cytokine production in the lungs in vivo as well as $T_H2$ cytokine production from T cells restimulated in vitro. Our data thus support the idea that these kinases have overlapping functions.

Although Txk is normally expressed at very low levels in $T_H2$ cells, our observations with these transgenic mice nonetheless help provide a better understanding of the specific functions of Txk and Itk which would have been otherwise difficult to address. In addition, these observations provided greater insight about the relevance of selective expression of Txk and Itk in $T_H1$ and $T_H2$ cells respectively. Our work also confirms previous studies by Berg and colleagues, as well as Luban and colleagues that show that, Txk expression is specifically downregulated in $T_H2$ cells (17,25). In contrast, these groups observed similar levels of Itk expression in $T_H1$ as well as $T_H2$ cells. Thus negative regulation of Txk expression may occur in a $T_H2$ specific manner, perhaps via negative regulation by $T_H2$ specific transcription factors. Alternatively, Itk's expression may be specifically maintained by $T_H2$ specific transcription factors. In either case, this expression pattern could result in a critical dependence of $T_H2$ cells on the function of Itk. Our work here lends support to this model since expression of Txk using the T cell specific CD2 promoter would allow for continuous expression of Txk, even in differentiating $T_H2$ cells, and thus provide crucial Tec kinase signals needed for functional responses in the absence of Itk. Hence, our observations suggest that the selective expression of Txk and Itk in $T_H1$ and $T_H2$ cells respectively may provide the delicate balance of signals required for inducing or maintaining different types of T cell responses. It should be noted that although the CD2-*Txk* transgene has been reported to improve positive selection in $Itk^{-/-}$ thymocytes, we find that it does not rescue peripheral T cell numbers (perhaps because the low T cell numbers are secondary to earlier defects in $Itk^{-/-}$ thymocytes (26). These observations suggest that the rescue of $T_H2$ responses are not merely the result of increased $CD4^+$ T cell numbers, although we cannot rule out other effects on development (3,5,27,28)). However, the normal levels of TCR-induced $Ca^{2+}$ influx in *Tg(CD2-Txk) and Tg(CD2-Txk)*$Itk^{-/-}$ peripheral T cells also argues that our results are not the result of non-specific hyperactivation of cells expressing the Txk transgene.

In previous studies, we have found that while the absence of Itk leads to defective $T_H2$ responses, absence of both Txk and Itk surprisingly leads to normal $T_H2$ responses in $Rlk^{-/-}Itk^{-/-}$ mice (8). Although these results may appear to contradict our current findings, it is possible that these findings result from the fact that $CD4^+$ T cells lacking both Txk and Itk maintain high levels of GATA3 following stimulation compared to WT and Itk null mice. These results suggested that a defect in GATA3 downregulation may lead to a propensity to develop into $T_H2$ cells in T cells from the Txk/Itk double knockout mice, perhaps because this was the only type of response that could occur. Our data presented here indicates that expression of Txk at similar levels as Itk can lead to normalized $T_H2$ responses in Itk-deficient mice, and that potential redundancy in function between Txk and Itk may explain the $T_H2$ specific response observed in *Tg(CD2-Txk)*$Itk^{-/-}$ mice.

Our data are also in support of recent data from Fowell and colleagues, who demonstrated that Itk deficient T cells can differentiate into $T_H2$ cells, however, they cannot elaborate and secrete $T_H2$ cytokines upon restimulation (19). The enforced expression of Txk in these $T_H2$ cells allows for functional rescue of this event. Given previous results suggesting that rescue of calcium signaling can rescue $T_H2$ cytokine production in *Itk*$^{-/-}$ CD4$^+$ T cells (11,19), it is likely that rescue of $Ca^{2+}$ mobilization by expression of the Txk transgene contributes to our observations. Thus, Txk may be able to rescue $T_H2$ responses by restoration of intracellular $Ca^{2+}$ increases that are defective in *Itk*$^{-/-}$ T cells, perhaps by participating in the Slp76/GADS/LAT complex, which regulate PLCγ1 activation. Indeed, expression of the Txk transgene has also been shown to be able to rescue PLCγ1 tyrosine phosphorylation in *Itk*$^{-/-}$ double positive thymocytes (13).

Other cell types such as mast cells and eosinophils have also been suggested to play an important role in the development of $T_H2$ specific responses (29-31). These cells are capable of producing $T_H2$ cytokines and chemokines that can induce initial immune responses and mediate further activation and migration of $T_H2$ cells to the lungs thereby exacerbating the immune responses (32). However, since the CD2 promoter cassette that drives Txk expression in these transgenic mice is primarily active in T cells, (including NKT cells), these findings suggest that the defect in Itk-null mice in developing effective $T_H2$ responses in our models is unlikely to be due to the lack of expression of Itk in other cell types such as mast cells (33).

In recent years there has been a growing interest in the Tec kinases Itk and Txk as potential therapeutic targets for $T_H2$ and $T_H1$-mediated diseases, respectively. Our data here support the model where the Tec kinases, Txk and Itk regulate T helper cell mediated responses via their differential expression in $T_H1$ and $T_H2$ cells respectively and not due to intrinsic functional differences as previously suggested (12-16). Our data also suggest that the defective $T_H2$ response in the absence of Itk is due to reduced Tec kinase signals, and that either Itk or Txk can fulfill this role if expressed at high enough levels. Overall, these findings provide novel insight into the role of Tec kinases Txk and Itk in the regulation of T helper cell differentiation/function and disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| AHR | Airway Hyperresponsiveness |
| BAL | Bronchoalveolar Lavage |
| PH | Pleckstrin Homology |
| PI3 Kinase | Phosphoinositide-3-kinase |
| SEA | Schistosome Egg Antigen |
| TCR | T cell Receptor |

## References cited


1. August A, Sadra A, Dupont B, Hanafusa H. Src-induced activation of inducible T cell kinase (ITK) requires phosphatidylinositol 3-kinase activity and the Pleckstrin homology domain of inducible T cell kinase. Proc Natl Acad Sci USA 1997;94:11227–11232. [PubMed: 9326591]
2. Debnath J, Chamorro M, Czar M, Schaeffer E, Lenardo M, Varmus H, Schwartzberg P. rlk/TXK encodes two forms of a novel cysteine string tyrosine kinase activated by Src family kinases. Mol Cell Biol 1999;19:1498–1507. [PubMed: 9891083]
3. Broussard C, Fleischecker C, Horai R, Chetana M, Venegas A, Sharp L, Hedrick S, Fowlkes B, Schwartzberg P. Altered development of CD8+ T cell lineages in mice deficient for the tec kinases Itk and Rlk. Immunity 2006;25:93–104. [PubMed: 16860760]
4. Schaeffer EM, Broussard C, Debnath J, Anderson S, McVicar DW, Schwartzberg PL. Tec Family Kinases Modulate Thresholds for Thymocyte Development and Selection. J. Exp. Med 2000;192:987–1000. [PubMed: 11015440]
5. Atherly L, Lucas J, Felices M, Yin C, Reiner S, Berg L. The Tec family tyrosine kinases Itk and Rlk regulate the development of conventional CD8+ T cells. Immunity 2006;25:79–91. [PubMed: 16860759]
6. Liao X, Littman D. Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity 1995;3:757–769. [PubMed: 8777721]
7. Berg L, Finkelstein L, Lucas J, Schwartzberg P. Tec family kinases in T lymphocyte development and function. Annu Rev Immunol 2005;23:549–600. [PubMed: 15771581]
8. Schaeffer E, Yap G, Lewis C, Czar M, McVicar D, Cheever A, Sher A, Schwartzberg P. Mutation of Tec family kinases alters T helper cell differentiation. Nat Immunol 2001;2:1183–1188. [PubMed: 11702066]
9. Ferrara T, Mueller C, Sahu N, Ben-Jebria A, August A. Reduced airway hyperresponsiveness and tracheal responses during allergic asthma in mice lacking tyrosine kinase inducible T-cell kinase. J Allergy Clin Immunol 2006;117:780–786. [PubMed: 16630934]
10. Mueller C, August A. Attenuation of Immunological Symptoms of Allergic Asthma in Mice Lacking the Tyrosine Kinase ITK. J Immunol 2003;170:5056–5063. [PubMed: 12734350]
11. Fowell D, Shinkai K, Liao X, Beebe A, Coffman R, Littman D, Locksley R. Impaired NFATc Translocation and Failure of Th2 Development in Itk Deficient CD4 T Cells. Immunity 1999;11:399–409. [PubMed: 10549622]
12. Suzuki N, Nara K, Suzuki T. Skewed Th1 responses caused by excessive expression of Txk, a member of the Tec family of tyrosine kinases, in patients with Behcet's disease. Clin Med Res 2006;4:147–151. [PubMed: 16809408]
13. Sommers C, Rabin R, Grinberg A, Tsay H, Farber J, Love P. A role for the Tec family tyrosine kinase Txk in T cell activation and thymocyte selection. J Exp Med 1999;190:1427–1438. [PubMed: 10562318]
14. Nagafuchi H, Takeno M, Yoshikawa H, Kurokawa M, Nara K, Takada E, Masuda C, Mizoguchi M, Suzuki N. Excessive expression of Txk, a member of the Tec family of tyrosine kinases, contributes to excessive Th1 cytokine production by T lymphocytes in patients with Behcet's disease. Clin Exp Immunol 2005;139:363–370. [PubMed: 15654836]
15. Takeno M, Yoshikawa H, Kurokawa M, Takeba Y, Kashiwakura J, Sakaguchi M, Yasueda H, Suzuki N. Th1-dominant shift of T cell cytokine production, and subsequent reduction of serum immunoglobulin E response by administration in vivo of plasmid expressing Txk/Rlk, a member of Tec family tyrosine kinases, in a mouse model. Clin Exp Allergy 2004;34:965–970. [PubMed: 15196287]
16. Takeba Y, Nagafuchi H, Takeno M, Kashiwakura J, Suzuki N. Txk, a member of nonreceptor tyrosine kinase of Tec family, acts as a Th1 cell-specific transcription factor and regulates IFN-gamma gene transcription. J Immunol 2002;168:2365–2370. [PubMed: 11859127]
17. Miller A, Wilcox H, Lai Z, Berg L. Signaling through Itk Promotes T Helper 2 Differentiation via Negative Regulation of T-bet. Immunity 2004;21:67–80. [PubMed: 15345221]
18. Hwang E, Szabo S, Schwartzberg P, Glimcher L. T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science 2005;307:430–433. [PubMed: 15662016]

19. Au-Yeung B, Katzman S, Fowell D. Cutting edge: Itk-dependent signals required for CD4+ T cells to exert, but not gain, Th2 effector function. J Immunol 2006;176:3895–3899. [PubMed: 16547221]
20. Schaeffer E, Debnath J, Yap G, McVicar D, Liao X, Littman D, Sher A, Varmus H, Lenardo M, Schwartzberg P. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. Science 1999;284:638–641. [PubMed: 10213685]
21. Ewart S, Levitt R, Mitzner W. Respiratory system mechanics in mice measured by end-inflation occlusion. J Appl Physiol 1995;79:560–566. [PubMed: 7592218]
22. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben T, Karp C, Donaldson D. Interleukin-13: central mediator of allergic asthma. Science 1998;282:2258–2261. [PubMed: 9856949]
23. Cohn L, Elias J, Chupp G. Asthma: mechanisms of disease persistence and progression. Annu Rev Immunol 2004;22:789–815. [PubMed: 15032597]
24. Fischer A, Mercer J, Iyer A, Ragin M, August A. Regulation of CXC chemokine receptor 4-mediated migration by the Tec family tyrosine kinase ITK. J Biol Chem 2004;279:29816–29820. [PubMed: 15123627]
25. Colgan J, Asmal M, Neagu M, Yu B, Schneidkraut J, Lee Y, Sokolskaja E, Andreotti A, Luban J. Cyclophilin A regulates TCR signal strength in CD4+ T cells via a proline-directed conformational switch in Itk. Immunity 2004;21:189–201. [PubMed: 15308100]
26. Lucas J, Felices M, Evans J, Berg L. Subtle defects in pre-TCR signaling in the absence of the Tec kinase Itk. J Immunol 2007;179:7561–7567. [PubMed: 18025201]
27. Hu J, August A. Naive and innate memory phenotype CD4+ T cells have different requirements for active Itk for their development. J Immunol 2008;180:6544–6552. [PubMed: 18453573]
28. Hu J, Sahu N, Walsh E, August A. Memory phenotype CD8(+) T cells with innate function selectively develop in the absence of active Itk. Eur J Immunol 2007;37:2892–2899. [PubMed: 17724684]
29. Taube C, Wei X, Swasey C, Joetham A, Zarini S, Lively T, Takeda K, Loader J, Miyahara N, Kodama T, Shultz L, Donaldson D, Hamelmann E, Dakhama A, Gelfand E. Mast cells, Fc epsilon RI, and IL-13 are required for development of airway hyperresponsiveness after aerosolized allergen exposure in the absence of adjuvant. J Immunol 2004;172:6398–6406. [PubMed: 15128831]
30. Lee J, Dimina D, Macias M, Ochkur S, McGarry M, O'Neill K, Protheroe C, Pero R, Nguyen T, Cormier S, Lenkiewicz E, Colbert D, Rinaldi L, Ackerman S, Irvin C, Lee N. Defining a Link with Asthma in Mice Congenitally Deficient in Eosinophils. Science 2004;305:1773–1776. [PubMed: 15375267]
31. Humbles A, Lloyd C, McMillan S, Friend D, Xanthou G, McKenna E, Ghiran S, Gerard N, Yu C, Orkin S, Gerard C. A Critical Role for Eosinophils in Allergic Airways Remodeling. Science 2004;305:1776–1779. [PubMed: 15375268]
32. Rothenberg M, Hogan S. The eosinophil. Annu Rev Immunol 2006;24:147–174. [PubMed: 16551246]
33. Zhumabekov T, Corbella P, Tolaini M, Kioussis D. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. J Immunol Methods 1995;185:133–140. [PubMed: 7665895]

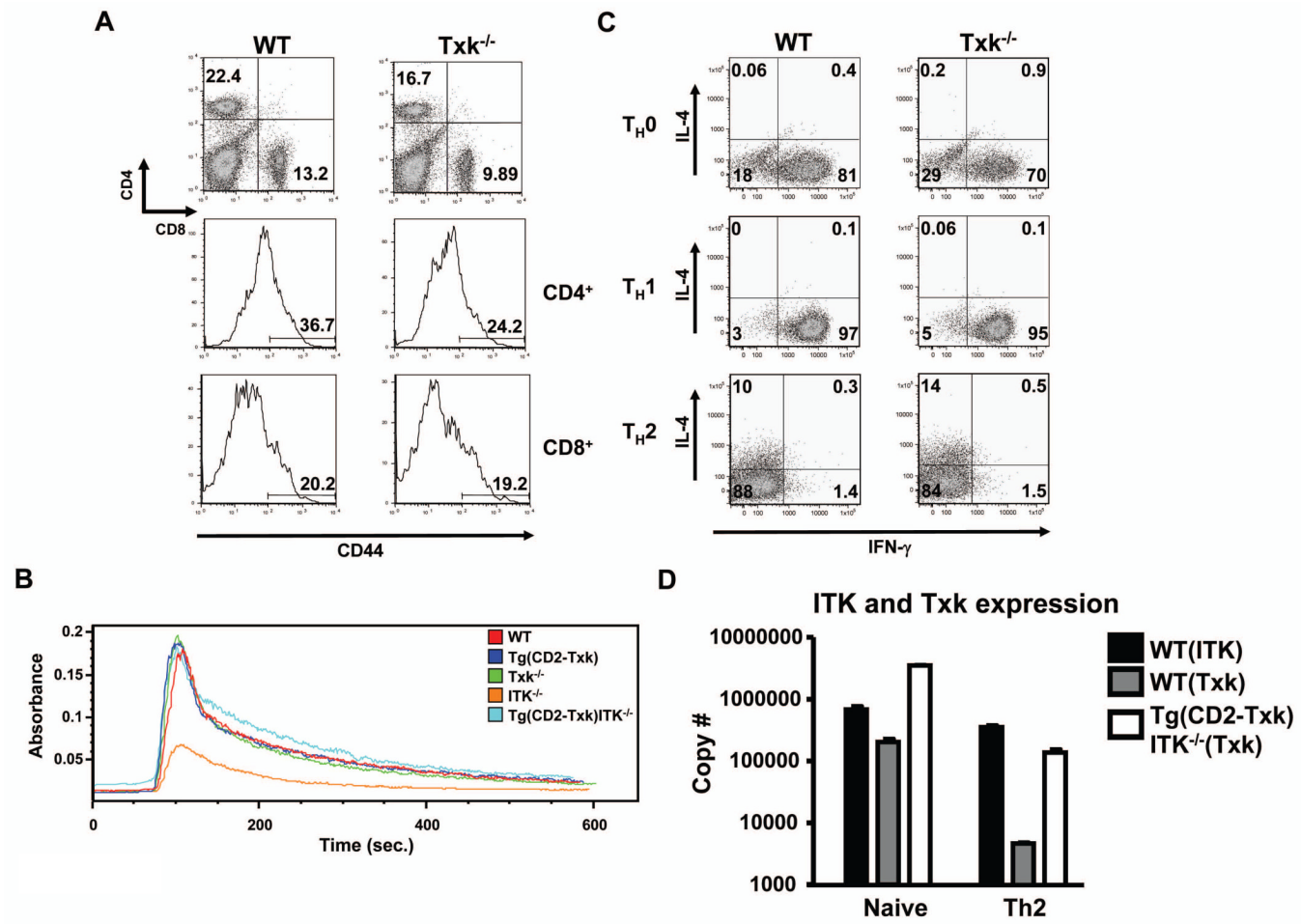


**Figure 1. Txk null T cells do not have defects in $T_H1$ or $T_H2$ cytokine secretion**

(a) Splenocytes from WT or Txk null mice were analyzed for the indicated markers by flow cytometry. Top panels: whole splenocytes; Middle panels: Gated $CD4^+$ T cells analyzed for CD44; Bottom panels: Gated $CD8^+$ T cells analyzed for CD44. (b) $CD4^+$ T cells from WT, Itk null, Txk null, *Tg(CD2-Txk)* or *Tg(CD2-Txk)Itk*$^{-/-}$ mice were analyzed for calcium response following stimulation with anti-CD3 antibodies as indicated. (c) Naïve $CD4^+$ T cells from WT or Txk null mice were differentiated under the indicated conditions, then restimulated and analyzed for expression of the indicated cytokines by intracellular staining and flow cytometry. Data are gated on $CD4^+$ T cells. (d) qRT-PCR of mRNA for Txk or Itk from fresh naïve $CD4^+$ T cells from WT or *Tg(CD2-Txk)Itk*$^{-/-}$ mice. Alternatively, these cells were differentiated under $T_H2$ conditions prior to qRT-PCR.

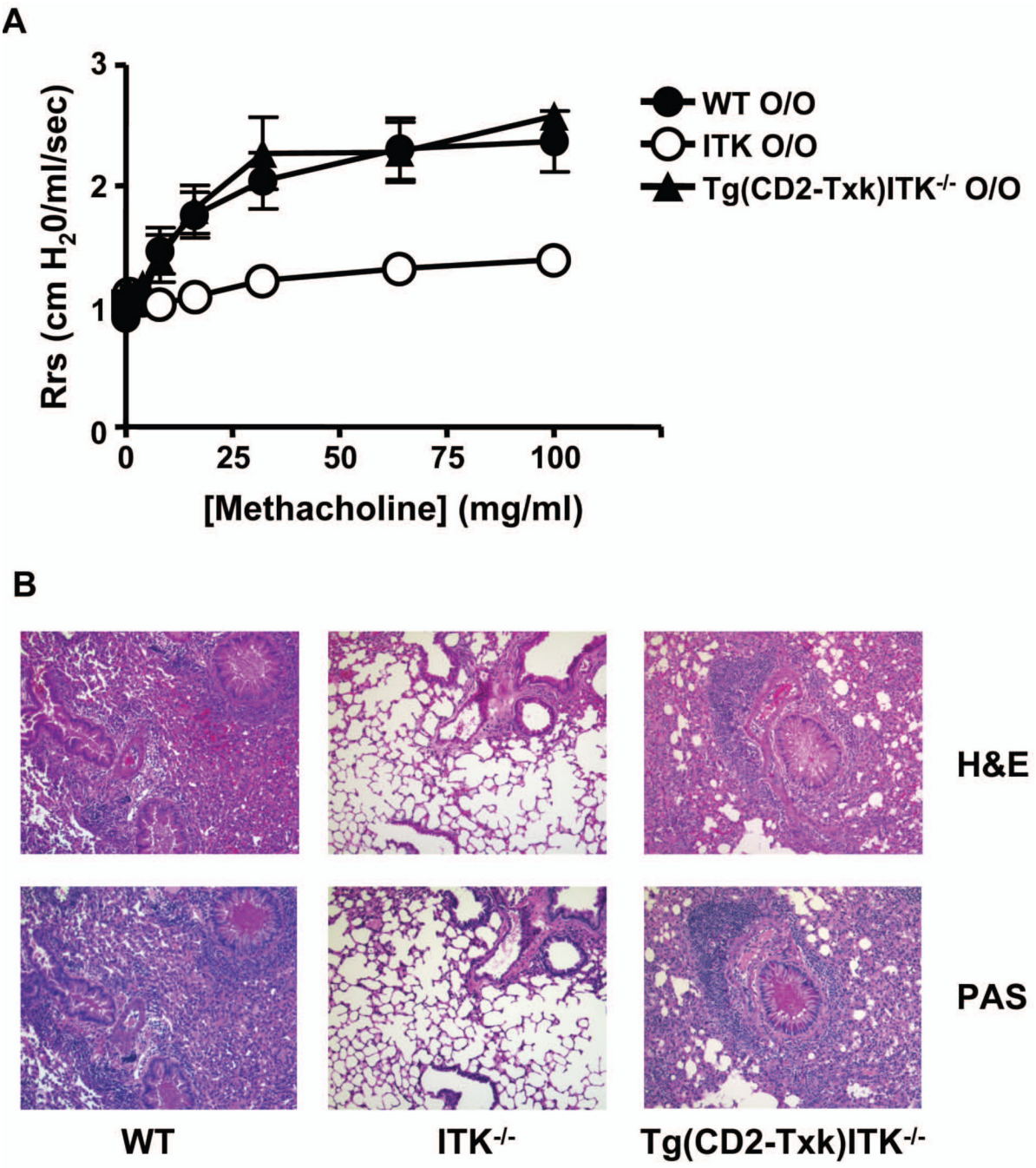


**Figure 2. Rescue of AHR in Itk-null mice by expression of the related kinase Txk**

(a) WT, Itk-null or *Tg(CD2-Txk)Itk*$^{-/-}$ mice were immunized and challenged intranasally with OVA, followed by analysis of AHR by mechanical ventilation. Filled circles, WT; open circles *Itk*$^{-/-}$; Filled triangles, *Tg(CD2-Txk)Itk*$^{-/-}$ mice. O/O indicates mice that have been immunized with OVA, then challenged intranasally with OVA. Differences are statistically significant between WT and *Itk*$^{-/-}$ or *Tg(CD2-Txk)Itk*$^{-/-}$ and *Itk*$^{-/-}$ ($p<0.05$) but not significant between WT and *Tg(CD2-Txk)Itk*$^{-/-}$ (n=5-7 mice per group, representative of three experiments)**.** (b) Mice were immunized and challenged intranasally with OVA, followed by analysis of lung sections by H&E (top panels) or PAS staining (bottom panels), representative of three experiments.

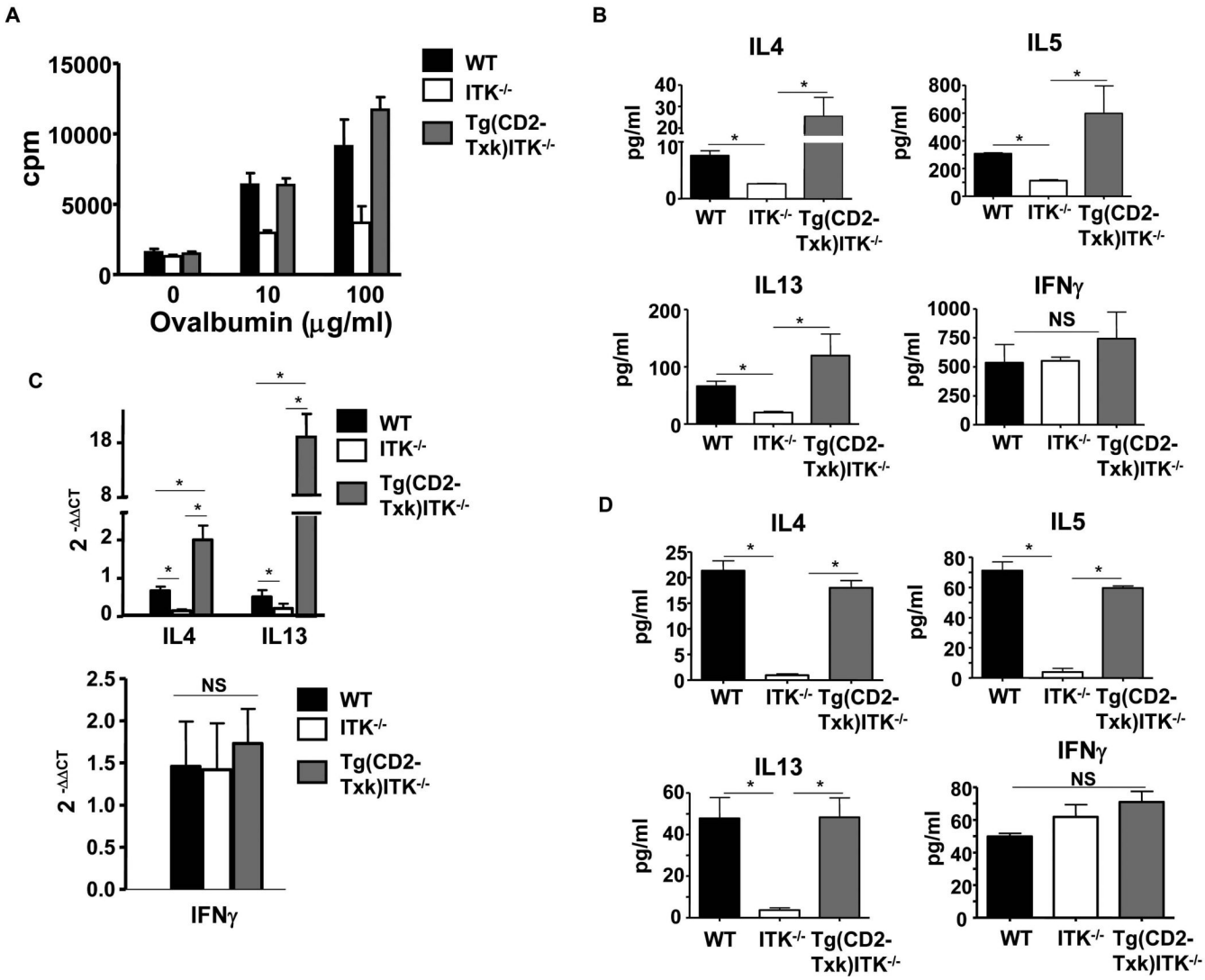


**Figure 3. Txk-mediated rescue of cytokine production in vitro and in vivo**

(a) Splenocytes from WT, Itk-null or *Tg(CD2-Txk)Itk*$^{-/-}$ mice treated as in figure 2 were incubated in vitro with the indicated concentration of OVA, and analyzed for proliferative responses after 96 h of culture. Data are the mean ± SEM of triplicate cultures and are representative of three experiments, *p<0.05. (b) Splenocytes were treated as in (a) with 100 μg/ml OVA and supernatants analyzed for IL-4, -5 and -13 and IFN-γ after 72 h of culture. Data are the mean ± SEM of triplicate cultures and are representative of three experiments, *p<0.05, NS, Not significant. (c) Lungs from WT, *Itk*$^{-/-}$ or *Tg(CD2-Txk)Itk*$^{-/-}$ mice treated as in figure 2 were analyzed by qRT-PCR for IL-4, IL-13 and IFN-γ. n = 6, *p<0.05, NS, not statistically significant. (d) BAL from lungs of mice treated as in figure 2 were analyzed for IL-4, -5, -13 and IFNγ. n = 6, *p<0.05, NS, not statistically significant.

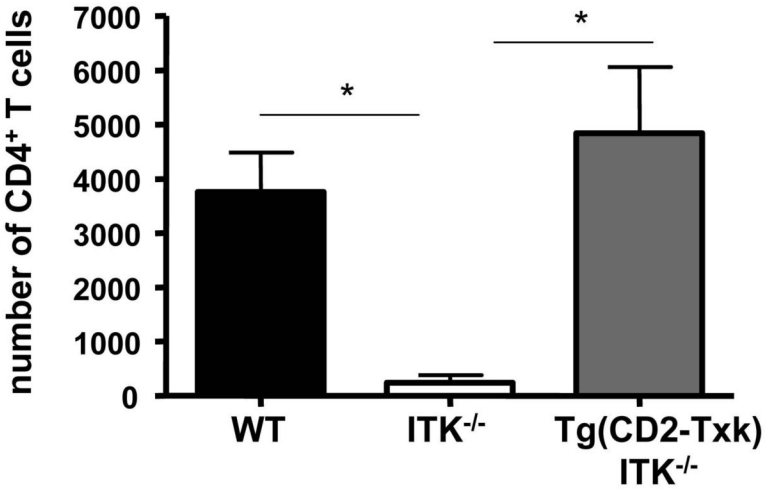


**Figure 4. Rescue of CD4$^+$ T cell recruitment into the lungs of Itk-null mice by expression of the related kinase Txk**

BAL fluid from lungs from WT, *Itk*$^{-/-}$ or *Tg(CD2-Txk)Itk*$^{-/-}$ mice treated as in figure 1 were analyzed for the number of CD4$^+$ T cells by flow cytometry. *$p<0.05$, $n = 6$, NS, not statistically significant.

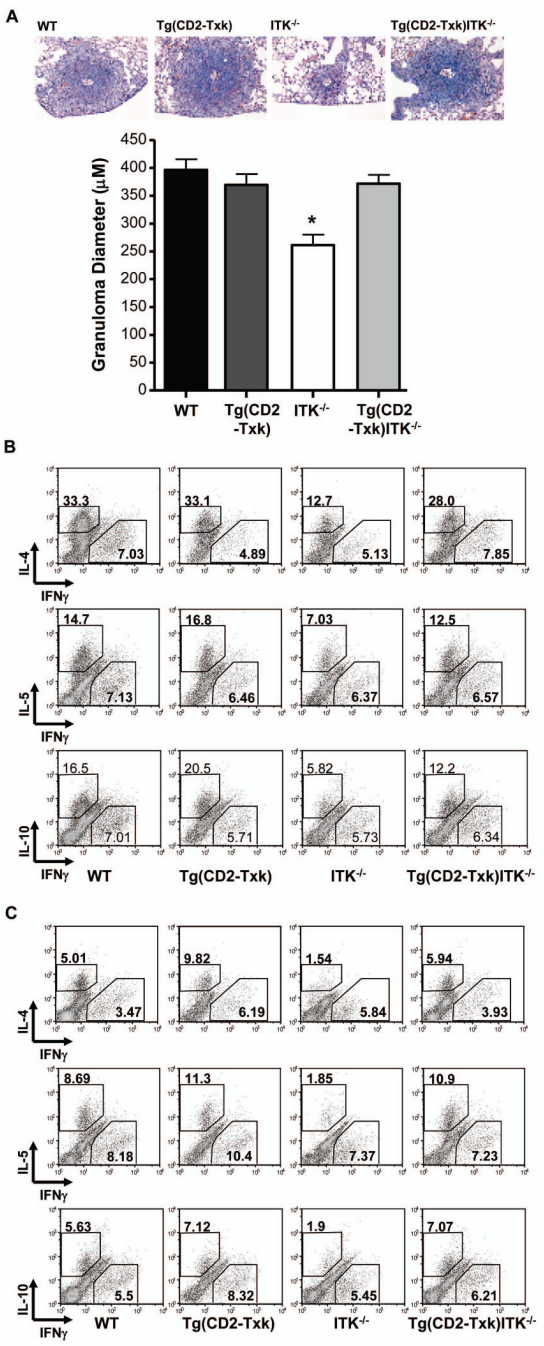


**Figure 5. Rescue of *S. mansoni* induced lung granulomas in Itk-null mice by Txk**

(a) WT, Itk-null, *Tg(CD2-Txk)* or *Tg(CD2-Txk)Itk*$^{-/-}$ mice were injected i.p., then challenged i.v. with *S. mansoni* eggs, followed by analysis of lung sections by H&E (top panel). Granuloma size was determined and plotted (bottom panel). Data are representative of two experiments examining 8-9 mice per genotype, *$p<0.05$. (b) Cells collected from the draining LN, or (c) spleens of mice treated as in Figure 5a were restimulated with SEA, fixed and analyzed for intracellular IL-4, -5, -10 and IFN-γ. Data are representative of two experiments each consisting 4-6 mice per genotype.