Role of Cybr, a cytohesin binder and regulator, in CD4+ T-cell function and host immunity

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**Abstract**

Cytohesin binder and regulator (Cybr) is known to regulate leukocyte adhesion and migration. However, its function in T-cells is poorly understood. Here, we investigated the role of Cybr in CD4+ T-cell function and host immunity. Cybr inhibited p38 phosphorylation following CD4+ T-cell stimulation. Since p38 regulates the expression of T-box expressed in T-cells (T-bet) but not GATA binding protein 3 (GATA-3) in T-cells, Cybr decreased the expression of T-bet and IFN-γ in CD4+ T-cells. Moreover, we found that host immunity against *Listeria* infection and IFN-γ production in blood were significantly compromised in Cybr-overexpressing transgenic mice. In summary, our data suggest that Cybr represses the expression of T-bet and IFN-γ via an inhibition of p38 in T-cells and consequently reduces host resistance to bacterial infection in mice.

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**1. Introduction**

Cybr, also known as B3-1, PSCDBP, Cytip and CASP, is expressed in T, NK, B and DC cells, in blood and spleen (Boehm et al., 2003; Dixon et al., 1993; Kim, 1999; Mansour et al., 2002; Tang et al., 2002). Its expression can be induced by cytokines (e.g., IL-12) and TCR stimulation in T-cells (Rogge et al., 2000; Tang et al., 2002). Cybr has been reported to associate with cytohesin and SNX27 (Boehm et al., 2003; MacNeil et al., 2007). Of note, Cybr acts as a scaffold protein in association with cytohesin family members and regulates guanidine nucleotide exchange activity of cytohesin proteins. This association is thought to reduce integrin-dependent T-cell adhesion and DC/T-cell interaction, leading to an increase in leukocyte migration (Boehm et al., 2003; Hofer et al., 2006; Mansour et al., 2002). The inhibitory role of Cybr in leukocyte adhesion has been confirmed in Cybr-deficient mice (Coppola et al., 2006). More recently, Cybr was proposed to be a signaling molecule in TCR pathways, resulting in the activation of NFAT and AP-1 through the JNK pathway in Jurkat cells (Chen et al., 2006). However, the same group showed no significant changes in T and DC activation and differentiation in Cybr-knockout mice (Watford et al., 2006). The discrepancy in the role of Cybr in T-cell activation and differentiation seen in the above cases was attributed to differences in the experimental systems and the genetic background of the knockout mice (Kolanus, 2007). Therefore, the role of Cybr in T-cells needs to be further clarified.

T-cells play an essential role in the maintenance of adaptive immunity. On encountering antigens, T-cells become activated, secrete cytokines and differentiate into functionally distinct effectors. IFN-γ is mainly produced by activated lymphocytes, namely T-cells and NK cells. One prominent function of IFN-γ is to activate macrophages, which, in turn, leads to the elimination of intracellular bacteria such as *Listeria* in cells or animals (Kaufmann, 1993). Accordingly, defects in the IFN-γ pathway are associated with susceptibility to disease caused by intracellular pathogens (Dorman and Holland, 2000). Proteins of the mitogen-activated protein (MAP) kinase family, ERK1/2, JNK and p38, are involved in T-cell development, activation and/or differentiation (Rincon, 2001), and also modulate IFN-γ expression in immune cells. For instance, p38 regulates the gene expression of T-bet and IFN-γ and Th1-cell differentiation in T-cells (Fukushima et al., 2005; Jones et al., 2003; Rincon et al., 1998), while ERK controls the induction of T-bet and IFN-γ expression in DCs and NK cells (Kondadasula et al., 2008; Li et al., 2006).

In this paper, we first generated two transgenic mouse lines which overexpress Cybr in T-cells. Next, we assessed the role of Cybr in the phosphorylation of MAP kinase family and the gene
expression of T-bet and IFN-γ in T-cells. Finally, we examined the effect of Cybr on serum IFN-γ production and host resistance to *Listeria* in mice.

### 2. Materials and methods

#### 2.1. Reagents and plasmids

Phorbol 12-myristate 13-acetate (PMA), ionomycin, ERK inhibitor (PD98059), anti-HA and anti-FLAG antibodies were purchased from Sigma–Aldrich (St. Louis, MO). JNK inhibitor (SP600125) and p38 inhibitor (SB202190) were purchased from Calbiochem (Madison, WI) and Alexis Biochemicals (San Diego, CA), respectively. Anti-p38, anti-phospho-p38, anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, and anti-phospho-JNK antibodies were from Cell Signaling Technology (Beverly, MA). Anti-T-bet, anti-GATA-3 and anti-Tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PARP, anti-CD3 and anti-CD28 were from BD Biosciences. Reporter constructs, pRL-TK, pB-gal-Luc and pGATA-3-Luc, have already been published (Chang et al., 2005; Yang et al., 2001). The Cybr transgenic vector, pmCD36–FLAG-Cybr, contained human Cybr cDNA with a trimeric FLAG driven by a T-cell-specific murine CD36 enhancer/promoter from a pGL3-mCD36/enhancer published elsewhere (Ji et al., 2002). Plasmid pME18S–FLAG-Cybr was obtained by in-frame sub-cloning a human Cybr cDNA to a pME18S–FLAG vector. Plasmid pCDNA3-HA-MKK6EE, which expresses a HA-tagged constitutively active mutant of MKK6, was described previously (Chen et al., 2003).

#### 2.2. Cells and mice

EL4 cells were obtained from the American Type Culture Collection. *Listeria monocytogenes* (BCRC 15386) was obtained from the Bioresource Collection and Research Center, Taiwan. Two transgenic lines (C56 and C51) of C57BL/6J mice, in which a FLAG-tagged human Cybr transgene is driven by the promoter and enhancer of the mouse CD36 gene, were generated in the transgenic core facility (Academia Sinica, Taiwan) and genotyped using PCR. Heterozygous transgenic mice were crossed with C57BL mice to generate heterozygous lines (C56 and C51) of C57BL/6J mice, in which a FLAG-tagged human Cybr transgene, were generated in the transgenic core facility (Academia Sinica, Taiwan) and genotyped using PCR. Heterozygous transgenic mice, were used to determine if a group was statistically significant from the control group, and were used as a template to perform real-time PCR using a primer set for the endogenous mouse Cybr mRNA (5’-GCCAATTG-GGACTCTGCTGTCG-3’ and 5’-GATCTGGCCCAAGCAACTGG-3’), total Cybr mRNA (mouse and transgenic human Cybr mRNA; 5’-GCAGCAATGGCAATTTG-3’ and 5’-GCAAAGCTGTCTTGTCGCCG-3’) or β-Actin mRNA (5’-CTCCCTCTGAGCGAACCTACTC-3’ and 5’-GGACTGCTCATACTCTCGTT-3’). Real-time PCR data were analyzed using an iQ5 multicolor real-time PCR detection system. Gene expression was normalized to the expression of β-Actin.

#### 2.6. Dual luciferase assay

EL4 cells (10 × 10^6) were electroporated at 975 μF and 260 V in a Bio-Rad Gene Pulser with pB-gal-Luc (10 μg) together with pRL-TK (1 μg), pME18S–FLAG-Cybr (0 or 25 μg) and pCDNA3-HA-MKK6EE (0 or 3 μg). These cells were stimulated with 6 h with vehicle (NS) or 100 ng/ml PMA plus 1 μM ionomycin. Ten micrograms of total lysate were used to determine the dual luciferase assays (Yang et al., 2001).

#### 2.7. Cumulative survival test

Six- to eight-week-old control or C56 mice were intraperitoneally infected with a dose (2 × 10^6) CFU/mouse) of *Listeria*. Daily death rate and number of bacteria in CFU per organ (spleen and liver) were determined.

#### 2.8. Statistical analysis

Data in the paper are representative of three independent experiments and are expressed as mean ± standard deviation unless indicated otherwise. For the survival data, the log-rank test was used to determine if a group was statistically significant from the control group. For the other experiments, ANOVA was performed to determine whether there was a significant difference between treatment groups and mock control groups. P < 0.05 (*) was considered to be statistically significant.

### 3. Results

#### 3.1. Generation and characterization of Cybr transgenic mice

Descriptions of the function of Cybr in Jurkat cells, a T-cell line, or primary T-cells of Cybr-deficient mice are not consistent (Chen et al., 2006; Coppola et al., 2006; Watford et al., 2006). Here, we re-examined the role of Cybr in T-cell functions by using a transgenic approach. T-cell-specific Cybr transgenic lines, C51 and C56, were generated (Fig. 1A) and we examined the expression level of Cybr transgene relative to endogenous Cybr in both lines. Real-time PCR data showed that the quantity of total (transgenic plus endogenous) Cybr mRNA in CD4+ T-cells of C51 mice was 3.3 times and of C56 mice was 7.1 times higher than endogenous Cybr mRNA in CD4+ T-cells of control as well as transgenic mice (Fig. 1B). Consistently, the ratio of the protein level of transgenic Cybr in CD4+ T-cells of C51 to C56 mice was 1:3, which is close to the relative levels of transgenic Cybr mRNA (Fig. 1C). Hereafter, C56 mice were used through the whole study.
3.2. Effects of Cybr on phosphorylation of MAP kinase family and effects of p38 on T-bet expression in CD4+ T-cells

To investigate the impact of Cybr on T-cell activation, we first evaluated the phosphorylation of MAP kinase family in activated T-cells. As expected, anti-CD3 antibody stimulation increased the phosphorylation of p38, ERK1/2 and JNK in wild-type CD4+ T-cells in a stimulation-dependent manner (Fig. 1D). Of note, a decrease in p38 phosphorylation was observed in CD4+ T-cells of C56 mice (data not shown). ERK and p38 have been shown to regulate T-bet expression in T-cells, NK cells and DCs (Jones et al., 2003; Li et al., 2006). In our system, dual luciferase assays showed that T-bet transcription in CD4+ T-cells was repressed by p38 inhibitor SB202190 but not ERK inhibitor PD98059 (Supplementary Fig. 1). However, no MAPK inhibitors significantly affected GATA-3 (Supplementary Fig. 1). Furthermore, we tested wild-type CD4+ T-cells with SB202190 in the absence or presence of anti-CD3 antibody. We found that SB202190 at 25 μM halved levels of T-bet protein but had little effect on GATA-3 levels in T-cells (Fig. 2). These data suggest that p38 is the key MAP kinase for TCR-mediated T-bet up-regulation.

3.3. Cybr reduces expression of T-bet and IFN-γ in CD4+ T-cells and serum IFN-γ levels in mice

We found that Cybr suppressed p38 phosphorylation in CD4+ T-cells following T-cell activation and that p38 inhibition reduced T-bet expression following T-cell activation. These data raised the possibility that Cybr suppresses T-bet expression via p38 inhibition in T-cells. To test this hypothesis, we compared the expression of T-bet in CD4+ T-cells from wild-type and C56 mice. CD4+ T-cells from wild-type mice had higher T-bet mRNA levels than those from C56 mice (Fig. 3A). It was the case for nuclear and cytosolic T-bet protein levels (Fig. 3B). The above data support the concept that Cybr negatively regulates T-bet expression via p38. This concept was further supported by the discovery that a dominant-positive MKK6 (MKK6EE) abrogated the repression of T-bet transcription by Cybr in EL4 T-cells (Fig. 3C).

T-bet is required for optimal expression of IFN-γ in T-cells (Szabo et al., 2002; Usui et al., 2006). Therefore, we examined IFN-γ expression in CD4+ T-cells from wild-type and C56 mice. We showed that Cybr overexpression decreased IFN-γ mRNA levels (Fig. 3A) and IFN-γ production (Fig. 3D) in CD4+ T-cells. Moreover, we examined IFN-γ levels in wild-type and C56 mice infected with a sub-lethal dose of Listeria. In our case the serum IFN-γ levels in C56 mice were significantly lower than those in wild-type mice from day 2 to day 8 (Fig. 4). Overall, the in vitro and in vivo data indicated the inhibitory effect of Cybr on IFN-γ expression.

3.4. Cybr decreases host resistance to Listeria infection in mice

IFN-γ is crucial for macrophage activation and in turn, Listeria clearance. Therefore, we next studied the in vivo function of Cybr using Listeria as a model pathogen in control and C56 mice. All C56 mice infected with Listeria (2 x 10⁵ CFU) died, compared with only about half of wild-type control mice infected at the same dosage (Fig. 5A). Moreover, Listeria load 5 days post infection in the livers and spleens of C56 mice (4.6 x 10⁶ CFU/liver and 1.3 x 10⁶ CFU/spleen) was significantly higher than in control littersmates (1.8 x 10⁶ CFU/liver and 1.0 x 10⁶ CFU/spleen; Fig. 5B). The whole data show that Cybr reduces host resistance to Listeria infection in mice, which is consistent with its negative regulation of IFN-γ expression in T-cells. The likely mechanism of Cybr in T-cells is proposed in Fig. 5C.
Fig. 3. Effects of Cybr on T-bet and IFN-γ expression in CD4⁺ T-cells. (A) CD4⁺ T-cells, isolated from wild-type (WT) or C56 mice (C56), were stimulated with anti-CD3 antibody for 0, 24 or 48 h. The cells were divided into two aliquots. Total RNA was extracted from one aliquot and converted into cDNA. The cDNA was used as template for PCR reaction. The ratio of the expression level of T-bet or IFN-γ to that of GAPDH is indicated (upper panel). The same results are re-plotted as line charts in lower panel. (B) nuclear and cytosolic proteins, extracted from the other aliquot of CD4⁺ T-cells from (A), were immunoblotted with antibodies against T-bet, PARP or Tubulin. (C) EL4 cells were already transfected with p-T-bet-Luc plus pRL-TK, pME18S-FLAG-Cybr (Cybr) and pcDNA3-HA-MKK6EE (MKK6EE). These cells were stimulated with vehicle (NS) or PMA plus ionomycin (P+I). Total lysate was subjected to dual luciferase assays or Western blotting with anti-HA, anti-FLAG or anti-Tubulin antibodies. (D) CD4⁺ T-cells, isolated from wild-type (WT) or C56 mice (C56), were stimulated with anti-CD3 antibody (0.1, 0.5 or 1.0 µg/ml) or anti-CD3 antibody (1.0 µg/ml) plus anti-CD28 antibody (0.1 µg/ml) for 24 h. IFN-γ levels of the supernatants were measured using ELISA kits. P < 0.05 (*) is considered to be statistically significant.

4. Discussion

In the present paper, we showed that Cybr reduced p38 phosphorylation, expression of IFN-γ and T-bet, and Listeria protection in Cybr-transgenic mice. For the first time, we showed that Cybr plays a negative role in T-cell signaling, which is consistent with a negative role of Cybr previously shown in leukocyte migration (Coppola et al., 2006) or other pathways (Kolanus, 2007). Our experiments with Cybr-transgenic mice complement other studies of Cybr on T-cell function in Cybr-knockout mice, but argue against the conclusion from Cybr-knockout mice that Cybr is not essential for T-cell function, probably because of functional redundancy (Watford et al., 2006).

MAP kinase pathways are activated in TCR signaling (Halloran, 1999; Zhang et al., 1999). Cytohesin proteins are thought to be a positive regulator of MAP kinase family members such as ERKs in the signaling pathways downstream of LFA-1, and adenosine and insulin receptors (Kolanus, 2007). Cybr is presumed to bind cytohesin-1 and, in turn, sequester the interaction of cytohesin-1 and LFA-1, leading to a reduction of LFA-1 signaling (Boehm et al., 2003; Hofer et al., 2006). However, Cybr was reported to increase the phosphorylation of JNK and p38 but not ERK in Jurkat cells retrovirally infected with Cybr. Cybr augmented TCR signaling through JNK activation (Chen et al., 2006). In contrast, though our Cybr-transgenic mice data confirmed the role of Cybr in the phosphorylation of JNK and ERK in Jurkat cells, the Cybr inhibition of p38 phosphorylation in our case (Fig. 1D) is completely different from the increase of p38 activation by Cybr in Jurkat cells (Chen et al., 2006). The contradictory effect of Cybr on p38 activation between our data and other group may be due to the difference of leukemic
and solid lines show inhibition and promotion, respectively. A cumulative survival rate was determined. (B) the expression of T-bet and IFN-γ is considered to be statistically significant. (C) Schematic diagram describes the likely number (\( n \)) of mice used in each group is indicated in the parenthesis. \( P < 0.05 \) (*) is considered to be statistically significant. (C) Schematic diagram describes the likely pathway of Cybr in T-cells. Cybr inhibits p38 pathway and consequently, decreases the expression of T-bet and IFN-γ, leading to reduction of Listeria clearance. Dashed and solid lines show inhibition and promotion, respectively.

versus primary T-cells. ERK and p38 have been reported to augment T-bet expression in immune cells (Jones et al., 2003; Li et al., 2006). In T-cells, Cybr had no effect on ERK phosphorylation (Fig. 1D) in TCR/CD3 signaling, as published elsewhere (Chen et al., 2006). In contrast, p38 increases T-bet expression mediated by IL-27, IL-12 and TCR/CD28 in CD4+ T-cells (Jones et al., 2003; Koch et al., 2007; Owaki et al., 2006). Given an inhibitory function of Cybr in p38 phosphorylation, Cybr would be expected to suppress T-bet expression via p38 inhibition, which is consistent with our case (Fig. 3A and B). This notion is further corroborated by the finding that Cybr repression of T-bet transcription was abolished by MKK6EE, which is a specific activator of p38 pathway (Fig. 3C).

T-bet is required for optimal IFN-γ expression in T-cells (Szabo et al., 2002; Usui et al., 2006). IFN-γ signaling was also shown to enhance T-bet expression in lymphoid cells (Lighvani et al., 2001). We found here that Cybr decreases T-bet and IFN-γ in CD4+ T-cells. Is it possible that Cybr suppresses IFN-γ expression via p38 inhibition, leading to suppression of T-bet? Our data showing that Cybr could still decrease T-bet transcription in a T-cell line whose IFN-γ production is defective argue against the above scenario (data not shown).

In this study, we showed that Cybr decreased IFN-γ production in CD4+ T-cells via the p38/T-bet pathway. We also evaluated the effect of Cybr in CD8+ T-cells. Cybr decreased IFN-γ production in CD8+ T-cells to a lesser extent than in CD4+ T-cells (Supplementary Fig. 2). Moreover, we found that Th1 differentiation (i.e., percentage of IFN-γ-producing cells) in CD4+ T-cells from C56 mice was less than in CD4+ T-cells from wild-type mice (data not shown). Consistent with IFN-γ repression by Cybr in T-cells, serum IFN-γ levels in the transgenic mice were lower than those in wild-type mice (Fig. 4). Since IFN-γ cytokine is known to eliminate Listeria infection, the inability of Cybr transgenic mice to eliminate Listeria infection could be attributed to a decrease in IFN-γ production. Therefore, our results strongly suggest that Cybr decreases host immunity to Listeria infection by repression of IFN-γ production in T-cells.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.08.014.

**References**


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