

Published in final edited form as:

Matrix Biol. 2013 August 8; 32(6): 316–324. doi:10.1016/j.matbio.2013.02.009.

Thrombospondin-1 is a CD47-dependent endogenous inhibitor of hydrogen sulfide signaling in T cell activation

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Abstract

Thrombospondin-1 is a potent suppressor of T cell activation via its receptor CD47. However, the precise mechanism for this inhibition remains unclear. Because H₂S is an endogenous potentiator of T cell activation and is necessary for full T cell activation, we hypothesized that thrombospondin-1 signaling through CD47 inhibits T cell activation by antagonizing H₂S signaling. Primary T cells from thrombospondin-1 null mice were more sensitive to H₂S-dependent activation assessed by proliferation and induction of interleukin-2 and CD69 mRNAs. Exogenous thrombospondin-1 inhibited H₂S responses in wild type and thrombospondin-1 null T cells but enhanced the same responses in CD47 null T cells. Fibronectin, which shares integrin and glycosaminoglycan binding properties with thrombospondin-1 but not CD47 binding, did not inhibit H₂S signaling. A CD47-binding peptide derived from thrombospondin-1 inhibited H₂S-induced activation, whereas two other functional sequences from thrombospondin-1 enhanced H₂S signaling. Therefore, engaging CD47 is necessary and sufficient for thrombospondin-1 to inhibit H₂S-dependent T cell activation. H₂S stimulated T cell activation by potentiating MEK-dependent ERK phosphorylation, and thrombospondin-1 inhibited this signaling in a CD47-dependent manner. Thrombospondin-1 also limited activation-dependent T cell expression of the H₂S biosynthetic enzymes cystathionine β-synthase and cystathionine γ-lyase, thereby limiting the autocrine role of H₂S in T cell activation. Thus, thrombospondin-1 signaling through CD47 is the first identified endogenous inhibitor of H₂S signaling and constitutes a novel mechanism that negatively regulates T cell activation.

Keywords

Thrombospondin-1; CD47; Hydrogen sulfide; T lymphocytes; Extracellular signal-regulated kinase; Redox signaling

1. Introduction

Thrombospondin-1 (TSP1) is a large (450 kDa) matricellular glycoprotein that plays a pivotal role in regulating vascular homeostasis (Bauer *et al.*, 2010; Isenberg *et al.*, 2009), platelet activation (Isenberg *et al.*, 2008), angiogenesis (Carlson *et al.*, 2008; Miller *et al.*, 2009; Roberts *et al.*, 2012), and immunity (Lopez-Dee *et al.*, 2011). TSP1 mediates these

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activities by binding to other extracellular matrix components and growth factors, mediating activation of latent TGF- β 1 (Schultz-Cherry *et al.*, 1993; Sweetwyne *et al.*, 2012), and binding to at least 12 different cell surface receptors (Murphy-Ullrich *et al.*, 2012). These receptors include five integrins (Calzada *et al.*, 2004a; Calzada *et al.*, 2003; Calzada *et al.*, 2004b; Chandrasekaran *et al.*, 2000; Lawler *et al.*, 1988; Staniszewska *et al.*, 2007), CD36 (Dawson *et al.*, 1997), CD47 (Gao *et al.*, 1996), CD148 (Takahashi *et al.*, 2012), calreticulin/low density lipoprotein receptor-related protein-1 (LRP1) (Elzie *et al.*, 2004), proteoglycans (Feitsma *et al.*, 2000), and sulfatides (Guo *et al.*, 1992). Among these, TSP1 has the highest affinity for CD47, and this receptor is both necessary and sufficient for TSP1 to inhibit NO-cGMP signaling (Isenberg *et al.*, 2006).

TSP1 regulates T cell activation and function in a domain specific manner. Although TSP1 enhances some T cell actions via its N-terminal domains, such as α 4 β 1 integrin-dependent adhesion and chemotaxis (Li *et al.*, 2002), the dominant effect of soluble TSP1 is the potent inhibition of TCR-mediated T cell activation (Li *et al.*, 2001). This inhibition requires interaction of the C-terminal domain of TSP1 with a proteoglycan isoform of CD47 on the T cell surface (Kaur *et al.*, 2011; Li *et al.*, 2002). The inhibitory activity of TSP1 does not require β 1 integrins (Li *et al.*, 2002) and is independent of TGF β , based on resistance to TGF β -function blocking antibodies (Li *et al.*, 2001) and the inhibitory activity of a recombinant signature domain of TSP1 that lacks the TGF β binding and activation sequences in the type 1 repeats (Ramanathan *et al.*, 2011). Further evidence that CD47 ligation is sufficient to inhibit T cell activation derives from the inhibitory activity of some CD47 antibodies and CD47-binding peptides such as 7N3 (FIRVVMYEGKK), but not the corresponding control peptide FIRGGMYEGKK (Li *et al.*, 2001).

Despite this evidence that CD47 ligation is necessary and sufficient for inhibiting TCR-dependent T cell activation, the lack of a substantial cytoplasmic domain in CD47 for docking of downstream signaling molecules suggests that lateral interactions with other membrane proteins such as growth factor receptors, integrins, PLIC-1, Fas receptor, and SIRPs are generally required for its signaling functions (reviewed in (Soto-Pantoja *et al.*, 2013).

While the proximal intracellular targets of TSP1/CD47-mediated inhibition of T cell activation are not known, this inhibition occurs downstream of the TCR targeting linker for activated T cells (LAT) and Zap70, but upstream of NFAT activation (Li *et al.*, 2001). TSP1 regulates the activation of soluble guanylate cyclase by NO in Jurkat T lymphoma cells in a calcium-dependent manner (Ramanathan *et al.*, 2011), but this pathway cannot account for the broad effects of CD47 signaling on T cell activation as cGMP signaling is not reported to play a major role in T cell activation and is limited to T cell differentiation (Niedbala *et al.*, 2006).

H₂S is emerging as an important member of the gasotransmitter family that also includes NO and carbon monoxide (CO). At toxic environmental concentrations (>200 ppm), H₂S inhibits mitochondrial cytochrome c oxidase (Reiffenstein *et al.*, 1992). Lower nontoxic concentrations have physiological functions in neuromodulation ((Abe *et al.*, 1996) and reviewed in (Tan *et al.*, 2009)), metabolic hibernation (Blackstone *et al.*, 2005; Blackstone *et al.*, 2007), protection from ischemia/reperfusion injury (Elrod *et al.*, 2007; Fu *et al.*, 2008; Jha *et al.*, 2008; Sivarajah *et al.*, 2006; Tripatara *et al.*, 2008), oxygen sensing (Olson *et al.*, 2009), vasodilatation (Hosoki *et al.*, 1997; Yang *et al.*, 2008), and promotion of angiogenesis (Wang *et al.*, 2010). Like its gasotransmitter cousins NO and CO, H₂S has transitioned from being perceived exclusively as toxin to recognition that it is an important endogenous signaling molecule. In common with NO, H₂S has been implicated as both a pro- (Bhatia *et al.*, 2005; Collin *et al.*, 2005; Cunha *et al.*, 2008; Zhang *et al.*, 2007) and anti-inflammatory

molecule in innate immune cells (Cunha *et al.*, 2008; Li *et al.*, 2007; Sivarajah *et al.*, 2009; Zanoardo *et al.*, 2006). Like NO, H₂S relies on its distinctive chemistry for signal transduction, which includes modification of specific protein cysteine residues (termed sulfhydration) and ligation of ferric iron, zinc, or copper centers in metalloproteins (Fukuto *et al.*, 2012).

Recently, we reported that H₂S is a potentiator of T cell activation in primary murine and human T cells and T cell lines (Miller *et al.*, 2012). Exogenously added H₂S, at nanomolar physiological levels (Furne *et al.*, 2008; Shen *et al.*, 2012) enhances both polyclonal and antigen-specific T cell activation. Notably the capacity of T cells to endogenously make H₂S via cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) is turned on as a result of T cell activation. Suppression of CBS and CSE expression by siRNA inhibits T cell activation and T cell proliferation, which can be rescued by supplementation with exogenous H₂S. Therefore, H₂S signaling is a necessary component of T cell activation.

As there are no reported endogenous inhibitors of H₂S signaling, we sought to examine the effect of inhibitory TSP1 signaling through CD47 on H₂S-mediated T cell activation. TSP1 is a logical candidate for regulation of this pathway given its potent and broad T cell inhibitory effects and its potent regulation of NO gasotransmitter signaling.

2. Results

2.1. TSP1 null T cells are more sensitive to H₂S-potentiated activation

In order to examine the role of TSP1 in H₂S-dependent T cell activation, we compared the activation of WT and TSP1 null CD3⁺ murine T cells via plate-bound anti-CD3 and anti-CD28 antibodies in the presence of H₂S. Using IL-2 gene expression as a marker of T cell activation, we observed, as previously, that H₂S dose-dependently enhanced IL-2 expression in WT T cells by up to 4-fold over control activated cells not treated with H₂S (Fig. 1A). H₂S enhancement of IL-2 expression was greater at all H₂S concentrations in the activated TSP1 null CD3⁺ cells (175% at 50 nM, 150% at 500 nM of WT cells) (Fig. 1A). In addition to IL-2, CD69 expression levels were also elevated 4-fold in the presence of 500 nM H₂S in TSP1 null activated T cells (Fig. 1B). Likewise we examined the role of endogenous TSP1 on H₂S-dependent T cell proliferation (Fig. 1C). TSP1 null murine CD3⁺ T cells stimulated with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of H₂S for 72 hours had significantly greater proliferation at 10 and 100 nM doses of H₂S, suggesting that the presence of TSP1 limits H₂S signaling in activated T cells.

2.2. Exogenous TSP1 limits T cell responses to H₂S

The increased H₂S-dependent proliferation of TSP1-null CD3⁺ T cells was inhibited in a dose-dependent manner by addition of exogenous TSP1 (Fig. 2A). Proliferation of murine TSP1-null CD3⁺ T cells stimulated with plate-bound anti-CD3 and anti-CD28 antibodies in the presence of H₂S for 72 hours at 1% O₂ was inhibited back to untreated levels by supplementation of TSP1 at 2.2 (1 μ g/ml) and 22 nM. Thus, exogenous TSP1 reverses the enhanced proliferation phenotype of TSP1 null T cells.

To more directly assess the ability of exogenous TSP1 to inhibit T cell activation in the presence of H₂S, we assessed the induction of IL-2 mRNA. We previously reported that TSP1 inhibits IL-2 mRNA and protein expression in human T lymphoma cells induced by TCR signaling (Li *et al.*, 2001). Addition of 2.2 nM TSP1 significantly inhibited activation measured by induction of IL-2 mRNA in WT primary CD3⁺ murine T cells and that stimulated by addition of 500 nM H₂S (Fig. 2B). To determine the specificity of this inhibition, we examined fibronectin, a multidomain protein of comparable size that binds similarly to heparin and binds to two β 1 integrins on T cells that also recognize TSP1

(Yabkowitz *et al.*, 1993). In contrast to TSP1, fibronectin at the same concentration did not inhibit basal or activation-dependent IL-2 mRNA expression in the absence or presence of 500 nM H₂S. Despite sharing several receptor binding specificities with TSP1, fibronectin does not directly interact with two known functional receptors for TSP1 on T cells: CD47 and calreticulin/LRP1 (Li *et al.*, 2005; Li *et al.*, 2006; Li *et al.*, 2002).

2.3. TSP1 limits T cell responses to H₂S via CD47

Engaging a proteoglycan isoform of CD47 by TSP1 inhibits T cell activation (Kaur *et al.*, 2011; Li *et al.*, 2002), whereas engaging calreticulin/LRP1 mediates internalization of TSP1 and modulates T cell motility and integrin function (Li *et al.*, 2005; Li *et al.*, 2006a). To determine whether CD47 is necessary for the inhibitory activity of exogenous TSP1, we examined *in vitro* activated WT, TSP1 null, and CD47 null CD3⁺ T cells in the presence and absence of 500 nM H₂S (Fig. 3). We again measured T cell activation using plate-bound anti-CD3 and anti-CD28 antibodies and observed activation-induced IL-2 mRNA expression in all backgrounds. Baseline activation by anti-CD3 + anti-CD28 was inhibited by addition of 2.2 nM exogenous TSP1 in the WT cells but not significantly altered by the TSP1 in the two null strains. IL-2 mRNA was enhanced more than 2-fold in the presence of 500 nM H₂S, and the H₂S dependent enhancement of activation was significantly inhibited in the presence of 2.2 nM exogenous TSP1 in the WT and TSP1 null backgrounds but not in CD47 null T cells (Fig 3C). This indicated that CD47 is the necessary receptor for TSP1 to inhibit H₂S signaling. Remarkably, the addition of both H₂S and TSP1 to activated CD47 null T cells produced a further stimulation of IL-2 expression relative to H₂S alone. The positive effect of TSP1 on IL-2 expression in the absence of CD47 is consistent with our previous report that TSP1 similarly increases CD69 expression in CD47-deficient human T cells via an undefined receptor (Kaur *et al.*, 2011) and with the known positive effects of TSP1 receptors such as β 1 integrins and calreticulin/LRP1 on T cell functions (Burbach *et al.*, 2007; Li *et al.*, 2005).

2.4. CD47 ligation is sufficient for inhibition of T cell activation by H₂S

To confirm the role of CD47 and examine possible contributions of other TSP1 receptors such as calreticulin/LRP1, we co-incubated the H₂S-exposed CD3⁺ murine T cells with several functional peptides derived from TSP1. The peptide 7N3 (¹¹⁰²FIRVVMYEGKK¹¹¹²) is derived from the C-terminal domain of TSP1 and binds to CD47 (Gao *et al.*, 1994; Isenberg *et al.*, 2006). As with full-length TSP1, we observed an inhibition of activation-induced IL-2 mRNA expression and the further stimulation by H₂S in WT primary T cell in the presence of 1 μ M 7N3 (Fig. 4A). In contrast to 7N3, the calreticulin binding peptide Hep1 (¹⁷ELTGAARKSRRLVKGPD³⁵) from the N-terminal domain of TSP1 (Yan *et al.*, 2011) at 1 μ M further enhanced IL-2 expression in cells activated in the presence of 500 nM H₂S but did not significantly increase IL-2 expression in nonactivated cells. Similarly, an integrin-binding peptide 766 (⁸⁷LALERKDHS⁹⁶) derived from the N-terminal domain of TSP1 (Calzada *et al.*, 2004a) at 1 μ M enhanced IL-2 expression in activated cells treated with H₂S.

H₂S-dependent induction of IL-2 mRNA in the presence of the CD47 binding peptide 7N3 was significantly inhibited to below control levels in WT T cells, demonstrating that ligation of CD47 is sufficient to inhibit H₂S signaling. To confirm that CD47 is the necessary for the inhibitory effect of this CD47 ligand on H₂S signaling, we examined its effect on H₂S signaling in CD47 null murine CD3⁺ cells (Fig. 4C). Treatment of CD47 null cells with 7N3 did not inhibit activation due to H₂S. Rather, 7N3 potentiated IL-2 mRNA expression induced by CD3+CD28 ligation and that observed by addition of H₂S in the CD47 null cells, where we expected 7N3 to have no effect. However, CD47-independent activities of 7N3 and other CD47-binding peptides are well known (Barazi *et al.*, 2002; Tulasne *et al.*, 2001),

and the nonspecific positive effect observed in Fig. 4C are consistent with the ability of 7N3 to stimulate integrin-independent adhesion of T cells lacking CD47 (Barazi *et al.*, 2002) and the known positive role of integrin activation in T cell activation (Pribila *et al.*, 2004). Despite this off-target activity, these data combined with those in Fig. 3C establish that CD47 is the necessary receptor for TSP1 inhibition of H₂S signaling in T cells.

2.5. TSP1 inhibits H₂S-induced ERK1/2 phosphorylation in activated T cells

As ERK signal transduction is important in mediating T cell activation and is reported to be a target of H₂S signaling in other cell types and tissues (Li *et al.*, 2011), we examined the effect of H₂S on T cell activation-induced ERK signaling by monitoring time dependent ERK1/2 phosphorylation in the presence or absence of 300 nM H₂S (Fig. 5A,B). Activation of Jurkat T cells induced a robust increase in p-ERK1/2 as early as 5 min following activation. Temporally, this signal peaked at 5 min and declined at 10 and 15 min following activation. H₂S co-administration shifted the maximal p-ERK1/2 signal to 10 min. The H₂S-induced p-ERK1/2 signal was significantly higher at both 10 and 15 min than the control signal. To our knowledge, this is the first report of H₂S modulating ERK signaling at nanomolar levels.

To test whether increased ERK signaling contributes to H₂S-induced potentiation of T cell activation, we examined the activation of Jurkat cells in the presence of an inhibitor of MEK-mediated ERK phosphorylation, PD184161 (Fig. 5C). The TCR-stimulated enhancement of Jurkat cell activation by 300 nM H₂S assessed by induction of CD69 mRNA was significantly inhibited in the presence of 100 nM PD184161, suggesting that ERK signaling at least in part mediates the enhancement of T cell activation by H₂S.

Previously our lab reported that TSP1 signaling blocked angiogenesis in part by inhibiting NO-induced stimulation of ERK phosphorylation (Ridnour *et al.*, 2005). Here we examined whether TSP1 could also inhibit H₂S-dependent increases in T cell activation via suppression of ERK signaling. Due to the significant effect of H₂S 15 min following activation in Fig. 5A, we used this time point to examine the effect of TSP1. When human Jurkat T cells were preincubated with 2.2 nM TSP1 15 min prior to addition of H₂S, p-ERK1/2 levels were markedly decreased (Fig. 5D, E). Significantly, TSP1 pretreatment inhibited the H₂S-mediated enhancement of p-ERK1/2 levels.

We repeated the ERK activation experiments in CD47-deficient Jurkat T cells (clone JinB8 (Reinhold *et al.*, 1999) in order to further assess the role of CD47 in TSP1-mediated inhibition of H₂S signaling. H₂S-dependent ERK activation is intact in this cell line. However, TSP1 failed to inhibit H₂S-stimulated p-ERK1/2 levels (Fig. 5D,E). TSP1 alone elevated p-ERK1/2 in CD47-deficient but not in WT Jurkat cells, which suggests that additional TSP1 receptors mediate positive signaling through ERK and is consistent with our previous report that peptides from the central thrombospondin type 1 repeats activate ERK1/2 phosphorylation in Jurkat cells (Wilson *et al.*, 1999). Changes in CD69 mRNA expression in WT and CD47 null murine T cells paralleled the changes in ERK1/2 phosphorylation induced by H₂S and TSP1 (Fig. 5F). H₂S increased CD69 expression induced by CD3+CD28 ligation in WT and CD47 null cells, but TSP1 inhibited H₂S-induced CD69 expression only in the WT cells. Although CD47-mediated inhibition of H₂S signaling probably involves other pathways in addition to ERK1/2, these data further validate the role of CD47 as a mediator of TSP1-dependent inhibition of H₂S signaling in T cell activation and identify ERK1/2 as one of its targets.

2.6. TSP1 limits T cell activation-induced up-regulation of H₂S production

In addition to the role of exogenous H₂S, we previously showed that full T cell activation depends on expression of the endogenous H₂S-producing enzymes CBS and CSE (Miller *et al.*, 2012). As TSP1 is a potent inhibitor of T cell activation, we examined its role in the expression of endogenous H₂S biosynthetic enzymes as a function of T cell activation. Purified CD3⁺ T cells were activated using platebound anti-CD3 and anti-CD28, and cells were harvested at 24 hours to analyze the expression of CBS and CSE mRNAs. As previously reported, activated WT murine CD3⁺ T cells show an increase in expression of CBS and CSE mRNA when compared to non-activated cells (Fig. 6A,B). Remarkably, non-activated TSP1-null CD3⁺ cells showed 15- and 8-fold higher basal CBS and CSE mRNA expression, respectively, relative to WT cells (Fig. 6A,B). As in the wild-type cells, however, the expression level increased in TSP1 null cells with activation to levels significantly higher than in the non-activated control cells. Furthermore, CBS and CSE levels were higher in activated TSP1 null cells than in activated WT cells. This implies that endogenous TSP1 limits the H₂S biosynthetic capacity of both resting and activated T cells, possibly contributing to the inhibition of T cell activation by TSP1.

3. Discussion

Recently we reported that physiological levels of the gasotransmitter H₂S in the nanomolar range function as an endogenous potentiator of T cell activation (Miller *et al.*, 2012). The present work identifies an extracellular matrix signaling pathway that limits this H₂S function in T cells (summarized in Fig. 7). We demonstrate that the previously reported potent inhibition of T cell activation by TSP1 (Li *et al.*, 2001) is mediated at least in part through inhibiting T cell responses to H₂S and the H₂S biosynthetic capacity of T cells. To our knowledge, this is the first report of an endogenous inhibitory signaling pathway that limits H₂S signaling and expands the range of signaling functions controlled by the matricellular protein TSP1.

H₂S-dependent enhancement of primary murine CD3⁺ T cell activation and proliferation was increased in TSP1 null cells, but was reversed after the addition of exogenous TSP1, suggesting that endogenously produced TSP1 limits the effect of H₂S in the wild-type cells. The IC₅₀ dose of TSP1 based on the data in Figure 2A is somewhere between 0.22 and 2.2 nM. These levels of TSP1 are physiological in plasma and consistent with concentrations needed for the inhibition of NO-cGMP signaling via its high-affinity receptor CD47 (Isenberg *et al.*, 2006). We tested the hypothesis that CD47 is necessary and sufficient for the inhibition of H₂S signaling using CD47 null T cells and by replacing TSP1 with a CD47-binding peptide from the C-terminus of TSP1 (7N3), which was sufficient for inhibition of H₂S-dependent T cell activation. The specificity of this peptide was validated by using CD47 null CD3⁺ T cells in place of the wild-type T cells and lack of inhibitory activity for other functional TSP1 peptides that interact with different TSP1 receptors on T cells. The positive effects of peptide 7N3 and TSP1 on H₂S-dependent and -independent T cell activation in CD47 null cells are consistent with previous studies and mediated by yet to be identified receptors (Barazi *et al.*, 2002; Kaur *et al.*, 2011; Tulasne *et al.*, 2001).

While H₂S likely interacts with multiple signaling pathways to enhance T cell activation (Miller *et al.*, 2012), we examined the specific role of ERK signaling based on several reports of ERK activation by H₂S.

We found ERK phosphorylation to be rapid and transient in TCR-activated Jurkat cells, consistent with reports in other cell types. Interestingly, H₂S did not simply enhance p-ERK levels, but rather prolonged them. Where maximal phosphorylation occurred at 5 min or earlier in untreated cells, H₂S delayed this peak until at least 15 min. This prolongment may

indicate that H₂S enhances T cell activation by sustaining the ERK signaling pathway. TCR activation initiates the production of H₂O₂ that limits the activation of the ERK signaling pathway as a negative feedback mechanism (Devadas *et al.*, 2002). The inclusion of the exogenous antioxidants manganese [III] tetrakis (4-benzoic acid) porphyrin (MnTBaP) or N-acetylcysteine or the endogenous overexpression of peroxiredoxin II, all used to reduce O₂^{•-} and H₂O₂ levels, augmented p-ERK levels (Kwon *et al.*, 2003) and produced a qualitatively and temporally similar shift in ERK phosphorylation to that seen in our study, but with concentrations orders of magnitude higher than that of H₂S in this study. Perhaps H₂S enhances p-ERK levels by reversing or preventing inhibitory thiol oxidation of upstream signaling components responsible for attenuating p-ERK levels.

We previously showed that H₂S enhances the reorientation of the microtubule organizing center (MTOC) in T cells and enhances tubulin-dependent cell polarization (Miller 2012). Evidence from several labs indicates a connection between microtubule activity and ERK signaling. MTOC orientation to the virological synapse during the spread of Human T-lymphotropic virus type 1 (HTLV-1) is dependent on ERK signaling (Nejmeddine *et al.*, 2009). Likewise, ERK activity is necessary for NK cell MTOC reorientation and lytic activity (Chen *et al.*, 2007; Li *et al.*, 2008; Nejmeddine *et al.*, 2009). The actions of H₂S on the MTOC may be the result of its ability to activate ERK, warranting further investigation into this pathway. In this regard, TSP1 may limit the activity of tumor-infiltrating lymphocytes by down-regulating H₂S-dependent ERK signaling.

Interestingly in Fig. 5D,E we observed that TSP1 alone increases p-ERK levels in CD47 null cells. The concentration used (2.2 nM) is below what we should expect to elicit an effect through any known TSP1 receptor other than CD47. These data are consistent with the increased CD69 induction we previously reported when CD47-deficient Jurkat T cells were activated in the presence of TSP1 (Kaur *et al.*, 2011) and indicate that another unrecognized high affinity TSP1 receptor is present on T cells that has an opposing TSP1-dependent signal to CD47. Future studies in our lab will explore this unexpected result.

The increased CBS and CSE expression in TSP1 null CD3⁺ cells demonstrates that TSP1 is an in situ inhibitor of endogenous H₂S production. Interestingly we observed that these enzymes are more highly expressed in the non-activated TSP1 null CD3⁺ cells at 24 hours, and especially in the case of CBS, a less dramatic increase in expression is observed upon T cell activation. The expression of CBS and CSE is known to be highly dependent on SP1 promoter elements (Ge *et al.*, 2001; Maclean *et al.*, 2004; Yang *et al.*, 2011), and our data may reflect an elevation of SP1 activity in TSP1 null cells. As we also did not sort the CD3⁺ populations into memory and naïve T cells, the differences in non-activated expression of CBS and CSE in TSP1 and WT cells could reflect differences in T cell population subsets in the spleen and their differences in enzyme expression levels, although no reports of this yet exist. However, we previously found no difference in CD4⁺ versus CD8⁺ T cell percentages and no elevation in memory T cells in splenic populations from TSP1-null mice based on CD25 or CD69 expression and the percentage of CD44-high T cells (Kuznetsova *et al.*, 2005).

One pathophysiological condition in which TSP1 may provide a homeostatic role in limiting H₂S-dependent T cell signaling is inflammatory bowel disease (IBD). The absence of TSP1 increases the severity of experimental IBD in both acute and chronic models. TSP1 null mice develop colonic focal inflammation after only two days of dextran sodium sulfate exposure in experimental colitis (Punekar *et al.*, 2008). In a mouse model of chronic colitis using multiple cycles of dextran sulfate exposure, the TSP1 null mice exhibit greater disease-associated angiogenesis and tissue inflammation (Zak *et al.*, 2008). Among the possible mechanisms for these findings is decreased immunosuppressive latent TGF-β

activation in TSP1 null mice. However, we would also postulate that given the enhanced level of H₂S associated with IBD, the lack of TSP1 could result in excessive H₂S stimulation of T cell activity.

A specific role of T cell CD47 as a TSP1 receptor in IBD has not been established, but this would be consistent with published evidence that CD47 null mice exhibit exaggerated T cell inflammatory responses (Bouguermouh *et al.*, 2008; Lamy *et al.*, 2007). In contrast, CD47-null mice exhibited a deficit in dendritic cell recruitment in a colitis model that was attributed to the function of CD47 as a counter-receptor for SIRP α (Fortin *et al.*, 2009). The balance between SIRP α -dependent and TSP1-dependent roles of CD47 in IBD will require further study.

4. Experimental Procedures

4.1. Cells and reagents

H₂S refers to any of its various protonation states ($\text{H}_2\text{S} \rightarrow \text{HS}^- + \text{H}^+ \rightarrow \text{S}^{2-} + \text{H}^+$) with HS⁻ being the predominant form at physiological pH (pK_a = 6.8). Na₂S and NaHS, the corresponding sodium salts of these anionic forms of H₂S, are considered H₂S donors at physiological pH and are used as sources of H₂S for this study. C57Bl/6 mice were anesthetized and sacrificed by cervical dislocation, and their spleens were harvested for T cell culture. The spleens were gently ruptured in a 40 micron cell strainer (BD Biosciences) and placed over a 50 mL Falcon tube using the back end of a 6 cc syringe plunger. The cells were rinsed through with basal RPMI (0.1% BSA) and centrifuged at 200×g for 5 minutes. CD3⁺ T cells were purified by using a pan T cell isolation kit II and MACS MS columns (Miltenyi Biotec) without prior red-cell lysis according to the manufacturer's protocol. The cells were resuspended in 10 ml of RPMI 1640 containing 10% FBS, glutamine and penicillin/streptomycin and plated in a flask for 30 min at 37°C and 5% CO₂. Care and handling of animals was in accordance with protocol LP-012 approved by the Animal Care and Use Committee of the National Cancer Institute. TSP1 was purified from human platelets as described here (Miller *et al.*, 2010). Fibronectin was isolated from human plasma by gelatin affinity chromatography as described (Negre *et al.*, 1994). TSP1 peptides 7N3 (¹¹⁰²FIRVVMYEGKK¹¹¹²), Hep1, and 766 were available from previous studies or purchased from Peptides International (Calzada *et al.*, 2004a). The MEK inhibitor PD184161 was purchased from Cayman Chemical.

Wild-type Jurkat T cells (E6.1, ATCC) were maintained at 2-5×10⁵ cells per ml in RPMI 1640 medium supplemented with glutamine, penicillin/streptomycin, and 10% FBS. Cells were maintained in culture for a maximum of 4 weeks. For cell activation studies, Jurkat cells were resuspended in basal medium (RPMI, glutamine, penicillin, streptomycin, and 0.1% BSA).

Unless specified otherwise, all chemicals were purchased from Sigma (St. Louis, MO). For T cell activation, wells of either 6-well or 96-well plates were coated overnight with a mixture of anti-CD3 and anti-CD28 antibodies (mouse cells: clones 17A2 and 37.51 respectively, BD Biosciences; Jurkat cells: OKT3 and CD28.2, functional grade, eBioscience) at 2 μg/ml and 5 μg/ml respectively for mouse cells and 2 μg/ml and 5 μg/ml in PBS without divalent cations. The following day, the wells were washed twice with PBS to remove unbound antibody, and cells were added in growth medium for stimulation.

The concentrations of H₂S used for this study are derived from the EC₅₀ concentrations of H₂S (from Na₂S) used for stimulation of T cell proliferation derived from our prior work (Miller *et al.*, 2012). Also, the steady state concentration of H₂S will depend on how much is added exogenously or made endogenously and the rate of its degradation. The degradation

of H₂S, both enzymatically and nonenzymatically, depends largely on O₂ concentration (Tiranti *et al.*, 2009). Minimizing the O₂ levels maximizes available H₂S. Due to this, we conducted the experiments at 1% O₂, 94% N₂ and 5% CO₂ unless stated otherwise.

4.2. Gene expression studies

In 6-well plates, 1 – 3×10⁶ cells for each condition were maintained in 1 or 20% O₂ for the specified amount of time (4-20 h). The cells were harvested and RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from 1 – 5 μg of total RNA using Superscript first strand RT-PCR reagents (Invitrogen) according to the manufacturer's protocol. qRT-PCR was then performed using the SYBR green kit (Thermo) on the following gene/primer sets: HPRT, IL-2, CD69, CSE, CBS (sequences can be found in (Miller *et al.*, 2012)). HPRT (hypoxanthine phosphoribosyltransferase) was used as the internal control for expression based on previous reports of its superior stability over other commonly used control genes (de Kok *et al.*, 2004). Results were calculated based on the delta-Ct method and normalized to HPRT.

4.3. T cell proliferation

Cells were seeded at 100,000 cells per well in 96 well plates uncoated or pre-coated with anti-CD3/CD28 as specified above and treated as indicated. Proliferation was assessed using cell titer-96 MTS reagent (Promega) after 72 hours of growth according to the manufacturer's protocol. The formazan signal produced by reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] inner salt by the cells on day 0 was subtracted from the signal after 72 hours to quantify net proliferation.

4.4. Western blotting

Jurkat cells were resuspended in fresh RPMI-G at 1×10⁶ cells per ml and 2 ml was added to activation plates and incubated at 37°C, 5% CO₂ for 5, 10, and 15 min. Cells were removed from the plates into cold 15 ml tubes and were pelleted at 300xg and resuspended in 100 μL of cold modified RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA with 1 mM NaF, 1 mM Na₃VO₄, and Proteoblock (Fermentas)). The cells then were sonicated for 5 min in a bath sonicator and lysates clarified by centrifugation at 16,000xg for 10 min at 4°C. Lysates then were processed for western blotting. Phospho-ERK1/2 was detected using an antibody from Cell Signaling (#9101) and total ERK with an antibody from Upstate (06-182), both diluted in 5% BSA TBST. Blots were developed with Amersham ECL Plus and imaged with a Kodak digital image station.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

Abbreviations

CBS	cystathionine β-synthase
CSE	cystathionine γ-lyase
ERK	Extracellular signal-regulated kinases
IL-2	Interleukin-2
MEK	Mitogen-activated protein kinase kinase

TCR	T cell antigen receptor
TSP1	Thrombospondin-1

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Highlights

- Endogenous and exogenous thrombospondin-1 limits the ability of H₂S to enhance T cell receptor-mediated T cell activation.
- CD47 is necessary for thrombospondin-1 to inhibit activation of T cells by H₂S.
- Thrombospondin-1 inhibits H₂S -induced ERK1/2 phosphorylation in activated T cells.
- Thrombospondin-1 limits activation-induced H₂S production by inhibiting expression of cystathionine β-synthase and γ-lyase.
- Thrombospondin-1 signaling through CD47 is the first identified endogenous inhibitor of H₂S signaling.

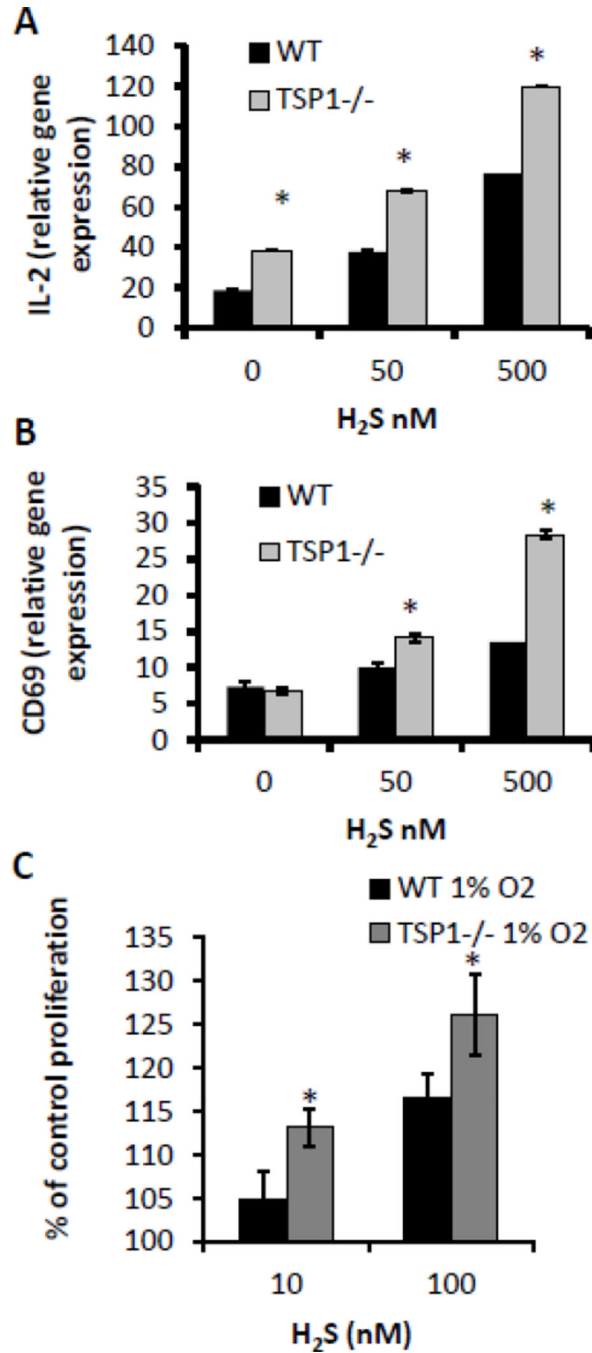


Figure 1.

H₂S-dependent potentiation of T cell activation and proliferation is enhanced in TSP1 null CD3⁺ cells. (A and B) Murine CD3⁺ T cells (3×10^6) were activated with plate-bound anti-CD3/CD28 antibodies in the presence of 50 or 500 nM Na₂S or vehicle in 1% O₂, and expression of IL-2 and CD69 mRNA was examined by RT-PCR at 4 hours respectively. Data are normalized to a value of 1 for non-activated control for each treatment, n=3, data are shown for a single experiment and are representative of n=2, error bars indicate standard deviation, * denotes $p < 0.05$. (C) TSP1 null and WT CD3⁺ cells were activated with plate-bound anti-CD3/CD28 antibodies in the presence of Na₂S or vehicle, and proliferation was assessed in a 1% O₂ atmosphere via an MTS assay at 72 hours post activation. Data

represent net proliferation relative to day 0 and are expressed as a percentage of untreated controls for cell type, n=3, error bars indicate standard deviation, * denotes $p < 0.05$ compared to vehicle control.

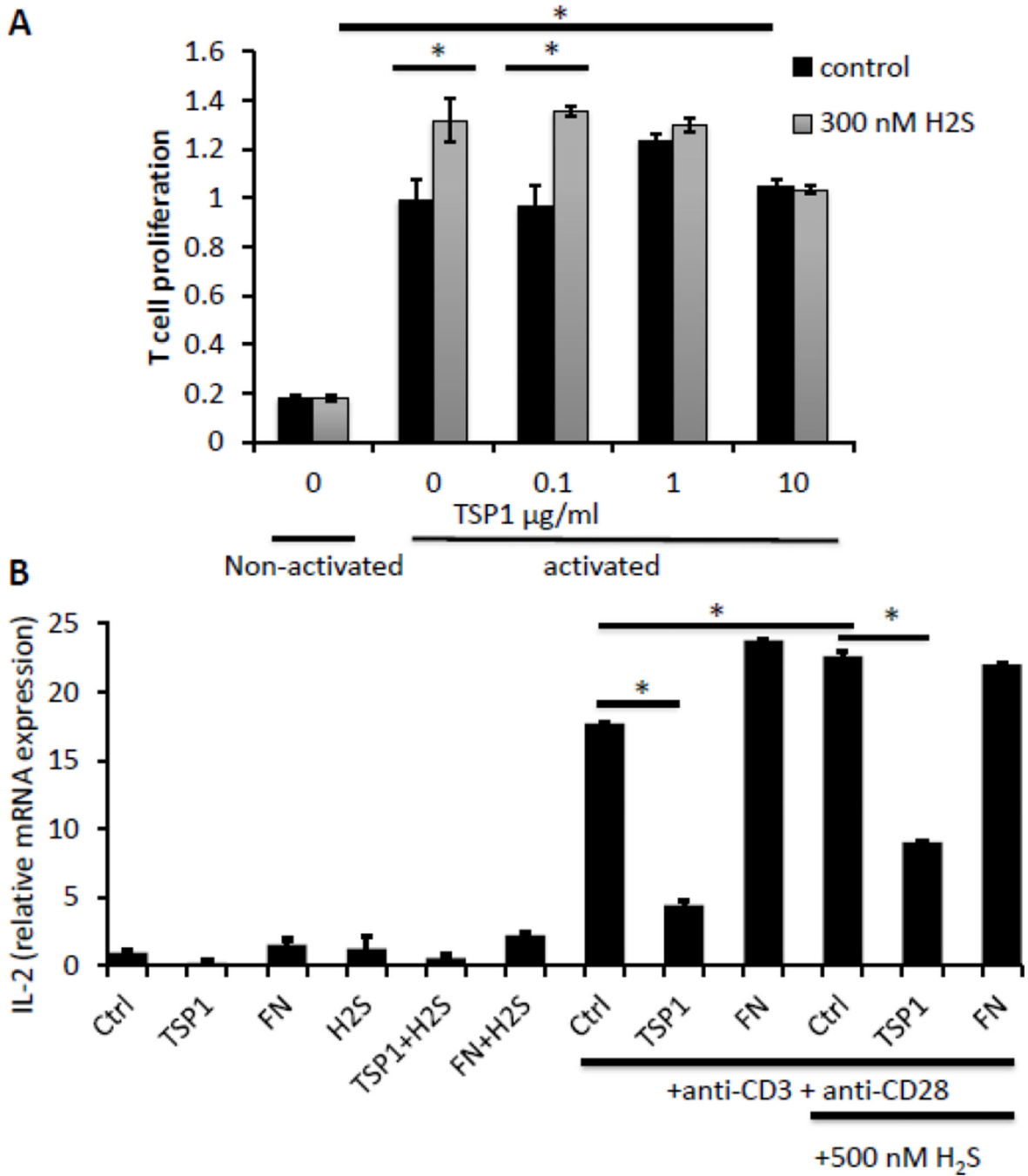


Figure 2. Exogenous TSP1 inhibits H₂S-dependent CD3⁺ T cell proliferation and activation. (A) TSP1 null and CD3⁺ cells were activated with plate-bound anti-CD3/CD28 antibodies in the presence of 100 nM Na₂S and TSP1 (0 to 10 µg/ml), and proliferation was assessed in a 1% O₂ atmosphere via an MTS assay at 72 hours post activation. Data represent net proliferation relative to day 0 and are normalized to 100% for untreated anti-CD3/CD28 activated controls for each treatment, n=3, error bars indicate standard deviation, * denotes p < 0.05 compared to vehicle control. (B) Murine CD3⁺ T cells were incubated without activation or on immobilized anti-CD3 +CD28 in the absence or presence of 500 nM H₂S and 1 µg/ml of TSP1 or fibronectin (FN). Cells were incubated for 20 h, and mRNA was isolated for

analysis of IL-2 mRNA expression. * denotes $p < 0.05$ for the indicated comparisons by ANOVA.

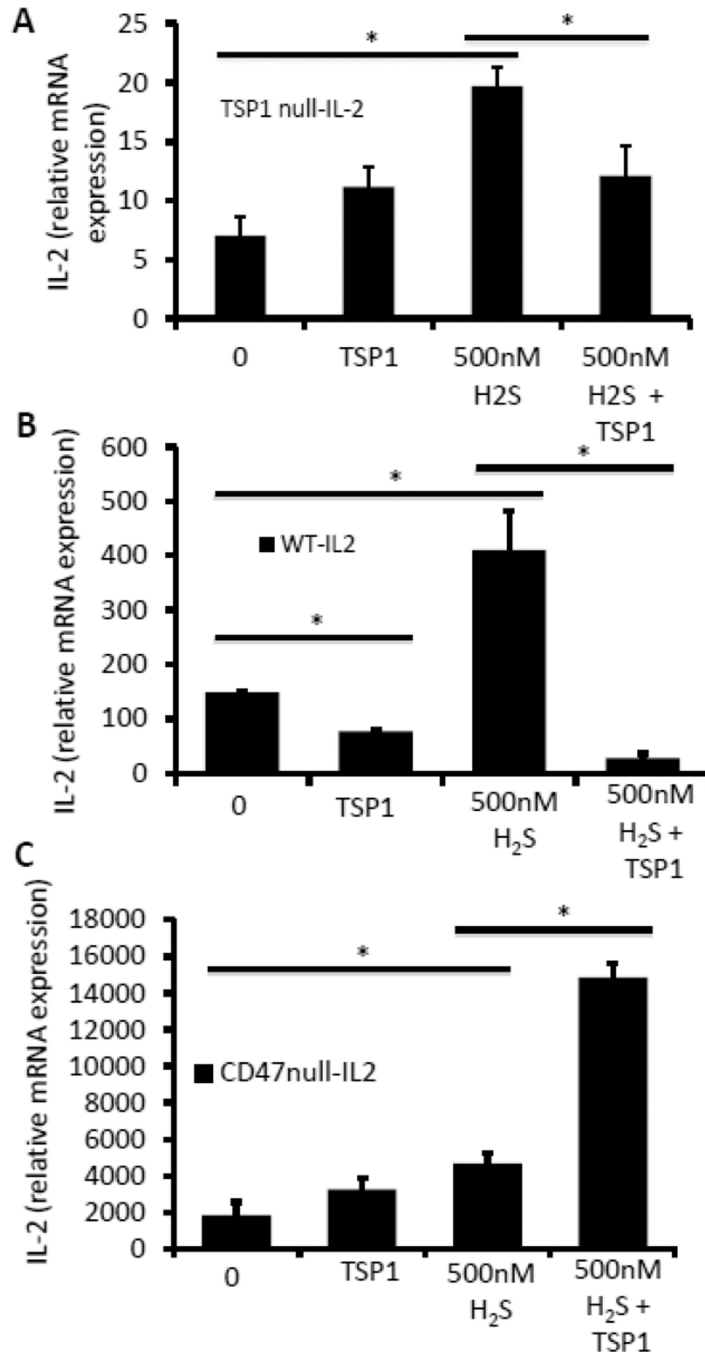
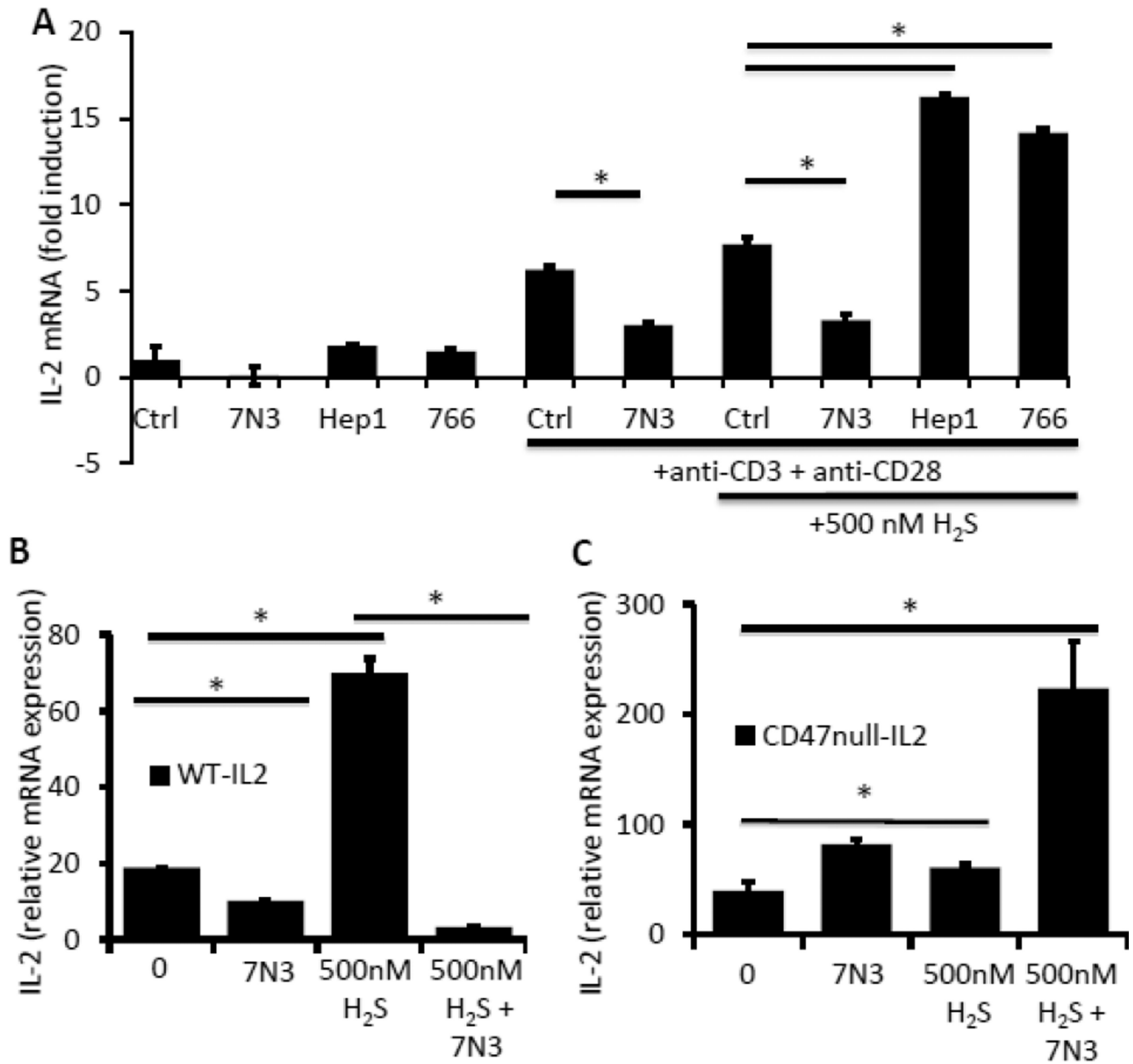


Figure 3. TSP1 inhibition of H₂S signaling is mediated by CD47. (A) Murine TSP1 null, (B) wild-type, or (C) CD47 null CD3⁺ T cells were activated with plate-bound anti-CD3/CD28 antibodies in the presence of 500 nM Na₂S, 2.2 nM of TSP1 or the combination in 1% O₂, and gene expression of IL-2 was examined by RT-PCR at 4 hours. Data are normalized to non-activated control for each treatment, and data are shown for a single experiment. Error bars indicate standard deviation, and * denotes p < 0.05 for the indicated comparisons using two way ANOVA or T test.

**Figure 4.**

Inhibition of H₂S signaling is mediated by CD47 binding peptide 7N3. (A) Murine WT CD3⁺ T cells were incubated in the absence or presence of immobilized anti-CD3 + CD28 and 500 nM H₂S in the presence of the indicated TSP1-derived peptides at 1 μ M. IL2-mRNA expression was measured after incubating for 20 h in 1% O₂. TSP1 peptides: CD47-binding peptide 7N3, calreticulin-binding peptide Hep1 (¹⁷ELTGAARKGSRRLVKGPD³⁵), and the integrin-binding peptide 766 (⁸⁷LALERKDHSG⁹⁶). (B) Murine wild-type or (C) CD47 null CD3⁺ T cells were activated with plate-bound anti-CD3/CD28 antibodies in the presence of 500 nM Na₂S, 1 μ M 7N3 or the combination in 1% O₂, and gene expression of IL-2 was examined by RT-PCR at 4 hours. Data are normalized to non-activated control for each treatment and data are shown for a single experiment. Error bars indicate standard deviation, and * denotes $p < 0.05$ for the indicated comparisons using two way ANOVA.

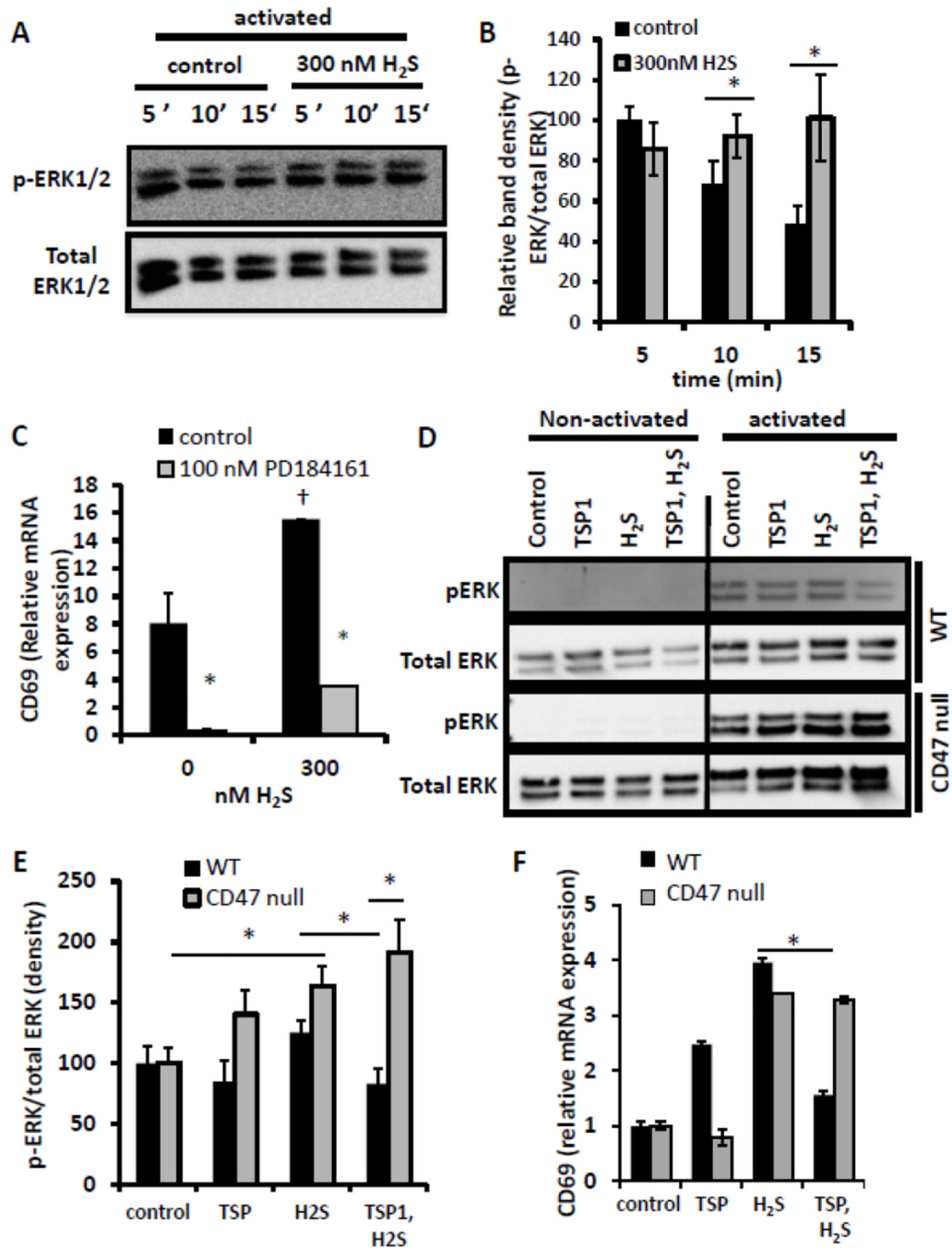


Figure 5. H₂S-mediated ERK phosphorylation is inhibited by TSP1 in a CD47 dependent manner. (A) Jurkat T lymphoma cells (2×10^6 cells) were activated with plate-bound anti-CD3/CD28 antibodies or control uncoated wells in the presence of 300 nM H₂S, and ERK phosphorylation levels were measured by Western blot at the indicated time points and compared to total ERK1/2 staining. (B) Graphical representation of band relative band density in A. (C) Jurkat cells (3×10^6 cells) were activated with plate-bound anti-CD3/CD28 antibodies in the presence of 300 nM Na₂S in 1% O₂, and expression of CD69 mRNA was examined by RT-PCR at 4 hours. Data are normalized to non-activated control for each treatment, n=3, error bars indicate standard deviation, * denotes $p < 0.05$ compared to cells

not treated with PD184161. † denotes $p < 0.05$ compared to cells not treated with H_2S . (D) Wild-type and CD47 null Jurkat T cells (2×10^6 cells) were activated with plate-bound anti-CD3/CD28 antibodies, control uncoated wells in the presence of 300 nM H_2S , 2.2 nM TSP1, or the combination, and ERK phosphorylation levels were measured by Western blot at 15 min and compared to total ERK1/2 staining. (E) Graphical representation of band relative band density in A expressed as a percentage of the respective untreated control cells. Western blots are representative of $n=2$. * denotes $p < 0.05$. (F) WT and CD47 null CD3+ T cells were activated on immobilized anti-CD3 plus anti-CD28 and treated with 2.2 nM TSP1 and/or 500 nM H_2S for 4 h. mRNA was isolated, and expression of CD69 mRNA was analyzed and is presented normalized to cells activated but not treated. * denotes $p < 0.05$ relative to cells treated with H_2S alone.

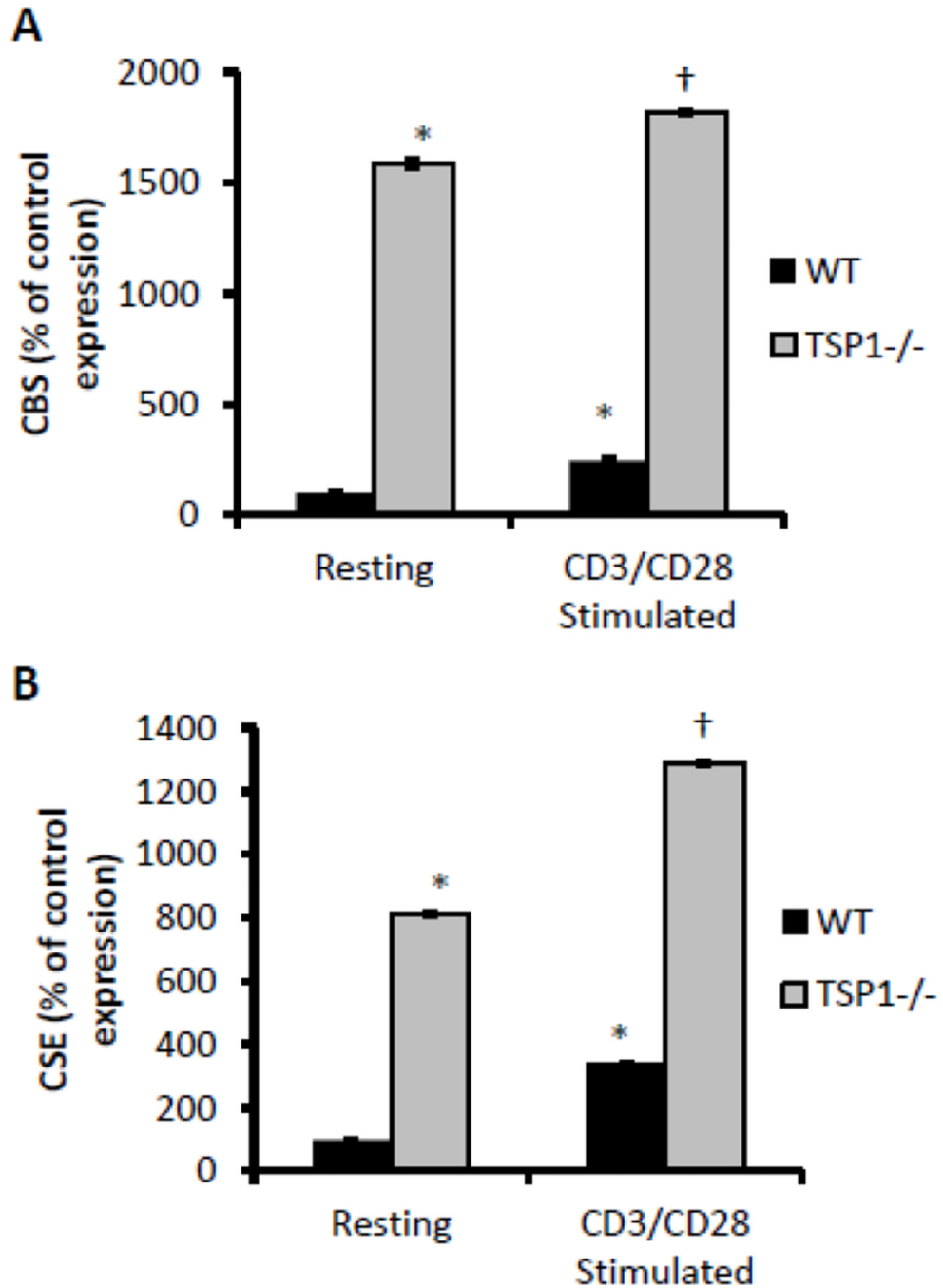


Figure 6.

H₂S biosynthetic capacity is unregulated in TSP1 null CD3⁺ T cells. Murine CD3⁺ T cells were activated with plate-bound anti-CD3/CD28 antibodies, and expression of CBS (A) and CSE mRNAs (B) were examined in 1% O₂ by RT-PCR at 24 h. Data are expressed as a percentage of non-activated control levels for each treatment; *n* = 3, data are shown for a single experiment and are representative of *n* = 2, error bars indicate S.D. * denotes *p* < 0.05 compared to resting wild-type control. † denotes *p* < 0.05 compared to resting TSP1 null control.

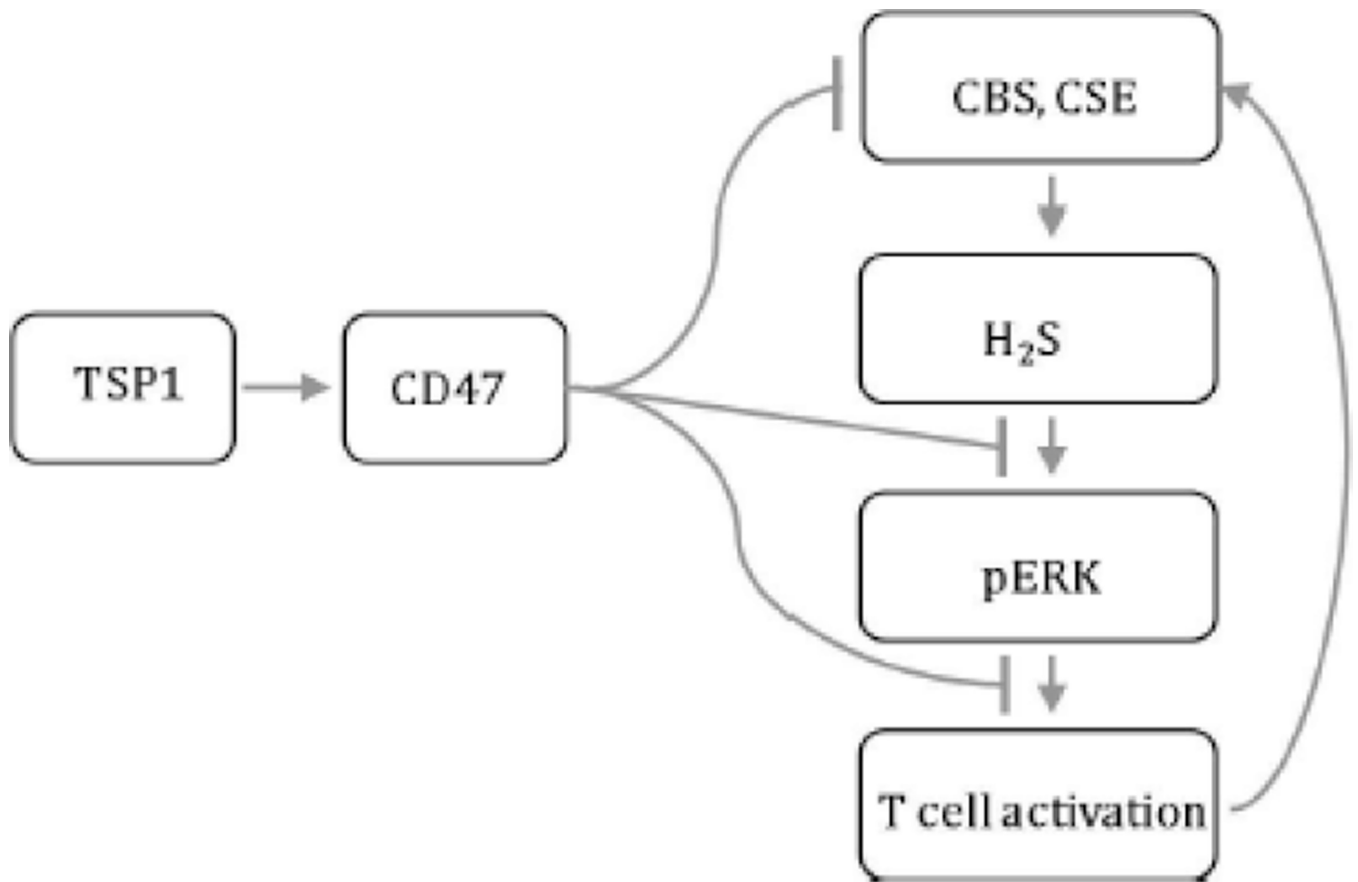


Figure 7. Schematic of TSP1 inhibition of H₂S-dependent T cell signaling through CD47. Exogenously added H₂S potentiates TCR-activated ERK phosphorylation to enhance T cell activation. T cell activation in turn stimulates the endogenous production of H₂S via transcriptional activation of its biosynthetic enzymes CBS and CSE. The secreted matricellular protein TSP1 engages its high affinity receptor CD47 on the surface of T cells to redundantly inhibit the H₂S signaling cascade. TSP1/CD47 signaling potently inhibits T cell activation via inhibition of H₂S-mediated ERK phosphorylation and also by limiting the expression of CBS and CSE. TSP1 is the first reported endogenous inhibitor of H₂S signaling.