Inducible, Dose-Adjustable and Time-Restricted Reconstitution of Stat1 Deficiency In Vivo

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Abstract

Signal transducer and activator of transcription (STAT) 1 is a key player in interferon (IFN) signaling, essential in mediating host defense against viruses and other pathogens. STAT1 levels are tightly regulated and loss- or gain-of-function mutations in mice and men lead to severe diseases. We have generated a doxycycline (dox)-inducible, FLAG-tagged Stat1 expression system in mice lacking endogenous STAT1 (i.e. Stat1ind mice). We show that STAT1 expression depends on the time and dose of dox treatment in primary cells and a variety of organs isolated from Stat1ind mice. In bone marrow-derived macrophages, a fraction of the amount of STAT1 present in WT cells is sufficient for full expression of IFN-induced genes. Dox-induced STAT1 established protection against virus infections in primary cells and mice. The availability of the Stat1ind1 mouse model will enable an examination of the consequences of variable amounts of STAT1. The model will also permit the study of STAT1 dose-dependent and reversible functions as well as of STAT1’s contributions to the development, progression and resolution of disease.

Introduction

Signal transducer and activator of transcription (STAT) 1 is involved in various cellular mechanisms such as proliferation, differentiation and apoptosis. It is part of the so-called Janus kinase (JAK)/STAT pathway, which is activated by the binding of interferons (IFNs) and other cytokines and growth factors to their cognate receptors. STAT1 is an essential component of the signaling pathways for all three types of IFN. IFN type I (mainly IFNαs and β) and IFN type III (IFNωs) activate the IFN stimulated gene factor 3 (ISGF3), which binds IFN stimulated response elements (ISRE). IFN type II (IFNγ) activates STAT1 homodimers, which bind to IFNγ-activated sites (GAS). Recruitment of STAT1 complexes to ISRE or GAS results in the induction of specific but overlapping sets of IFN induced genes (ISGs) [1–3].

In unstimulated cells, STAT1 generally resides in the cytoplasm in the form of inactive dimers. Activation of JAKs enables them to phosphorylate their receptors and thereby allow the recruitment of STAT proteins, which are subsequently phosphorylated on key tyrosine residues. Phosphorylation of STAT1 dimers on tyrosine 701 leads to a conformational change, translocation into the nucleus and initiation of transcription [4,5]. Additional phosphorylation on serine 727 takes place in the nucleus and is essential for full transcriptional activity [6–8].

Evidence is accumulating that the availability of STAT1 determines the responsiveness to IFN. Cellular STAT1 levels are maintained and enhanced through an autoregulatory loop that is dependent on IFN type I [9]. Accordingly, mice gene-targeted in components of the IFN type I production and response cascade (e.g. IFNβ, IFNα receptor 1 [Ifnar1], tyrosine kinase 2 [Tyk2], Stat2 and Ifi30) show reduced levels of STAT1 [10–13]. Knowledge of the basal amounts of STAT1 required for cellular functionality is sparse, although an increase of STAT1 in Ifnar1-deficient fibroblasts restores IFNγ-induced expression of target genes and antiviral activity [9]. It has also been shown that IFN type I-induced STAT1 levels counteract the STAT4-driven IFN γ production of activated NK cells [14]. The ratio of STAT1:STAT4 is also an important determinant of the CD8+ T cell response upon virus infection [15]. Analysis of Stat1-deficient T cells has revealed that STAT1 strongly effects the activation of Th1 lineage-specific enhancers, thus contributing to chromatin-shaping processes [16].

The importance of STAT1 is underlined by the effects of germline loss- and gain-of-function mutations in mice and men. Null mutations of Stat1 result in high susceptibility to bacterial and viral pathogens [17–21]. In humans, partial loss of STAT1...
activity leads to mycobacterial and viral diseases, although in contrast to cases completely lacking STAT1 such patients are curable [17,18,22–24]. Stat1-deficient mice kept under specific pathogen-free conditions do not succumb to infection and can be studied. STAT1 has been shown to have tumor-suppressive functions against spontaneous and induced solid cancers, including breast cancer [25–30]. In contrast, STAT1 has a tumor-promoting function in leukemogenesis [31] and progressed melanoma [32]. STAT1 gain-of-function mutations were discovered in human patients diagnosed with chronic mucocutaneous candidiasis caused by perturbed cytokine responses and resulting in impaired function of Th17 cells [33–38]. To date, gain-of-function studies of STAT1 in experimental systems have been restricted to the effects of the in vitro expression of constitutively active STAT1 (STAT1C) [39,40].

Genome-wide association studies reveal that common disease-associated polymorphisms are enriched in STAT1 promoter binding sites and that deregulated levels and/or activation of STAT1 are closely associated with the development of autoimmune/inflammatory disorders and cancers [41]. Consistently with this finding and the results of loss-of-function studies in mice [42], STAT1 expression is often lost in human breast cancer biopsies and STAT1 has been discussed as a predictive marker for breast cancer therapy [25,43]. Remarkably, STAT1 gain-of-function mutations and the concomitant increase in activity levels have been shown to distinguish disease phenotypes and outcomes in myeloproliferative neoplasms [44]. Balanced STAT1 activity is thus indispensable for functional immunity and prevention of tumor development in mice and men [18].

To date, there is no in vivo model available to study the impact of dose- and time-dependent STAT1 functions in a reversible manner. Tetracycline- or derivative-controlled gene expression systems have proven to be the ideal tool for reversibly inducible gene expression in mammals. We report here the generation of a doxycycline (dox) – inducible Stat1 (Stat1im) mouse model based on the Tet-On system [45]. We show that expression of inducible STAT1 depends on the time and dose of dox treatment in primary cells and tissues/organs. Detailed biochemical analysis proves that transgenic STAT1 can drive ISG expression and mediate antiviral activity in vitro and in vivo. Furthermore, we show that spatially and temporally balanced STAT1 expression is essential for host defense against virus infection.

Materials and Methods

Ethics Statement

Mice were housed under specific pathogen-free conditions according to FELASA guidelines. All animal experiments were discussed and approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna, conform to the guidelines of the national authority (the Austrian Federal Ministry for Science and Research) as laid down in §80f of the Animal Science and Experiments Act (Tiererversuchsgesetz – TVG; ref BMWF-68.205/0204-C/GT/2007; BMWF-68.205/0210-11/10b/2009, BMWF-68.205/0243-II/3b/2011) and according to the guidelines of FELASA and ARRIVE. To assess the distress of the animals during infection experiments, a scoring system was established. Based on this, health status and behavior of the animals were controlled by trained staff (participants of FELASA B training course) every 3–4 hours during 7 a.m. and 7 p.m. In order not to disturb the circadian rhythm of the animals there was no monitoring after 7 p.m. Human endpoint was conducted by cervical dislocation if death of the animals was to be expected during the following hours.

Cloning, Gene Targeting and Mice

For the establishment of Stat1im mice we used a 2nd generation Tet-On system based on a site-specific recombination system in embryonic stem (ES) cells [46,47]. The KH2 (C57BL/6×129Sv) ES cells express the M2 reverse tetracycline (tet)-controlled transactivator (M2rtTA) under the control of the endogenous Rosa26 promoter and a neomycin resistance cassette flanked by flipase recognition target (fl) sites inserted downstream of the collagen type I alpha 1 (Col1a1) locus. We targeted a construct carrying the tet operator (tetO) followed by a FLAG-tagged Stat1a cDNA (Genbank accession number NM_009205) into the Col1a1 locus of KH2 ES cells by fl/flipase-mediated recombination. Stat1a cDNA was PCR-amplified from C57BL/6N bone marrow-derived macrophages (BMMΦs) mRNA with the primers 5′ AGT GTC TCG AAT CCC CAT GCA ACA GTG GTA TCC TCC GCA TGG TCG GTA AGA CGT AGA CGT TAA TTA ATT AAG GTA CTT GTC GTC GTC CTT GTA ATC TAC TGT GCT CAT CAT ACT GTC ACA GTG GTT CGA and 5′ TGC TCG CAC GTA CTT CAT ACT GTC ACA GTG GTT CGA. The vector and a flippase recognition target (flp) element [46] and designated pBBS1-Stat1a-FLAG. The vector and a vector encoding the flipase (pCAGGS-FLAG) were co-transfected into KH2 ES cells using Nucleofector Technology (Ammaxa Inc.). ES cells were treated with 140 μg/ml hygromycin B (ROTH) to select clones that have undergone site-specific recombination, which converts the cells from G418- to hygromycin B-resistant. Three independent hygromycin B-resistant ES cell clones showed correct and single integration of the construct, as verified by Southern blot analysis (Fig. S1). ES cells were injected into C57BL/6N blastocysts and resulting chimeras were crossed to Stat1-deficient mice (B6N.129Stat1tm1Dlv, referred to here as Stat1−/−) [19]. We obtained triple-mutant mice heterozygous for (i) the M2rtTA in the Rosa26 locus, (ii) the dox-inducible Stat1 construct in the Col1a1 locus and (iii) the targeted mutation of the endogenous Stat1 locus. Heterozygous mice were intercrossed to obtain homozygous triple-mutant B6N.129P2-Stat1tm1Dlv; Gt(RoSA)26S{|Stat1mut;possStat1mut}tmalm; Col1a1tm1(Esp31);Stat1a−/− mice (referred to here as Stat1im mice). Wild-type (WT) mice and homozygous triple-mutant Stat1im mice were bred separately in a B6N;129Sv mixed genetic background. In parallel, mice were backcrossed to C57BL/6N and BALB/c by speed congenics [48]. All experiments were performed using WT and Stat1im mice in a B6N;129Sv mixed genetic background. In parallel, mice were backcrossed to C57BL/6N and BALB/c by speed congenics [48]. All experiments were performed using WT and Stat1im mice in a B6N;129Sv mixed genetic background and Stat1−/− mice in C57BL/6N. Mice were fed with doxycycline (dox; Sigma) at concentrations of 0.2 or 1 mg/ml supplemented with 10 mg/ml sucrose via drinking water.

Cell Culture and Reagents

ES cells, primary embryonic fibroblasts (PEFs) and BMMΦs were cultivated as previously described [49]. IFNβ and IFNγ were purchased from Calbiochem and Millipore. For in vitro experiments, cells were treated with dox dissolved in water at concentrations ranging from 25 ng/ml to 10 μg/ml.

PCR, RT-qPCR and Southern Blot

Genotyping of Stat1im mice and cells was performed using three different PCR protocols for each modified locus. Col1a1: Col1a1-fwd: 5′ TGC TCG CAC GCA GTA CTG CAT TC 3′; Col1a1-rev: 5′ CAA CCT GGT CCT CCA TCT GTT 3′; Hygro-fwd: 5′ ACT GTC GGG CCG ACA CAA AT 3′ (amplonc size for WT: 255 bp;
Survival experiments with mice using VSV and EMCV were performed as described [51]. Survival data were analyzed using Kaplan-Meier estimators.

Results

Time- and dose-dependent expression of doxycycline-induced STAT1 in ex vivo cultured cells from Stat1

STAT1 exists in two isoforms, full length STAT1α and STAT1β, which is truncated at the carboxy terminal and thus lacks part of the transactivation domain [52]. We used Stat1α-cDNA to generate inducible Stat1 mice. KH2 embryonic stem cells expressing the M2rtTA under the control of the endogenous Rosa26 promoter were targeted with a dox-inducible, FLAG-tagged Stat1α-cDNA downstream of the Col1a1 locus by flt/flipase-mediated site-specific recombination (Fig. 1A). Three independent clones showed correct and single integration of the construct, as proven by Southern blot analysis (Fig. S1). Transgenic STAT1 (termed STAT1FLAG) could be detected in all three ES cell clones after administration of dox for 24 h using a FLAG-tag specific antibody. Importantly, we did not observe any leakiness of the system, i.e. there was no STAT1FLAG expression in the absence of dox (Fig. S2A).

The gene-targeted ES cells were suitable for the generation of inducible Stat1-transgenic mice. ES cells were injected into C57BL/6N blastocysts and the resulting chimera were bred with Stat1-deficient C57BL/6N mice. Heterozygous offspring were intercrossed to obtain homozygous triple-targeted mice for (i) the tet-activator in the Rosa26 locus, (ii) the dox-responsive Stat1-construct and (iii) the targeted mutation in the endogenous Stat1 locus. Homozygous triple-targeted mice were designated Stat1ind and used for all experiments in a B6N:129Sv mixed genetic background with matching WT controls. Additionally, mice were backcrossed to C57BL/6N and BALB/c by speed congenics [48].

In the absence of dox, no STAT1FLAG could be detected in bone marrow-derived macrophages (BMMΦs) isolated from Stat1ind mice. STAT1FLAG expression was dependent on the dose of dox applied (Fig. 1B, upper panel). STAT1FLAG induction started after 2 h of dox treatment and reached WT levels after 24 h using 0.25 μg/ml dox (Fig. 1B, lower panel). STAT1FLAG was also expressed in primary embryonic fibroblasts (PEFs; Fig. S2B) and splenocytes (Fig. S2C) in a time- and dose-dependent manner. Only in PEFs was low basal STAT1FLAG detectable, indicating some degree of tissue-specific leakiness of the Col1a1 and/or Rosa26 loci. In contrast to previous attempts to generate inducible Stat1-transgenic mice [49], the Tet-On system gave rise to viable inducible Stat1 transgenics. Importantly, STAT1 levels similar to, above or below those of WT STAT1 can readily be achieved ex vivo, so the system is suitable for studying the dose effects of preformed STAT1 in various cell types.

STAT1FLAG is phosphorylated and binds to DNA upon IFN treatment in vitro

In dox-treated BMMΦs isolated from Stat1ind mice, STAT1FLAG was phosphorylated on tyrosine 701 and serine 727 at levels similar to WT STAT1 after 15 or 60 min treatment with IFNb or IFNγ (Fig. 2A). Electrophoretic mobility shift assays showed that in response to stimulation with IFNβ, the STAT1FLAG protein forms ISGF3 and binds to ISRE sites; in response to stimulation with IFNγ it forms STAT1 homodimers and binds to GAS sites. Again, DNA binding activity was comparable to that in WT cells (Fig. 2B). The data demonstrate the biochemical functionality of STAT1FLAG. In accordance with previous reports, the addition of short sequences to the C-terminus of

Western Blot and Electrophoretic Mobility Shift Assay (EMSA)

Protein lysates and Western blots were performed as described [50]. Additional antibodies were purchased from Bethyl Laboratories (ECS directed against the FLAG-tag) and Santa Cruz (β-tubulin). EMSAs were performed as described [49].

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded (FFPE) heart, liver and spleen tissue samples. Antigen retrieval was achieved by incubation in citric buffer pH 6 for 20 min at 100°C. For the detection of STAT1, rabbit polyclonal anti-STAT1 antibody (from Santa Cruz in Fig. 4; from Cell Signaling in Fig. S6) was applied. Antibody binding was visualized using peroxidase-conjugated secondary antibody.

Flow Cytometry

Freshly isolated splenocytes from WT and Stat1ind mice were stained for 20 min at 4°C with anti MHC class I-specific antibody H-2Db-PE in combination with anti-T cell-specific antibody CD4-PE in combination with anti-CD8-PE and anti-CD45RA-APC (eBioScience). For analysis, at least 5 × 10^4 cells were acquired and analyzed using FACSDivaTM software (both BD Biosciences). Mean fluorescence intensities were analyzed with linear models (ANOVA and t-tests) using genotype and treatment as factors and resulting p-values reported.
STAT1 does not affect the protein’s ability to dimerize, to be phosphorylated and to bind DNA [5,53].

**STAT1FLAG** is a fully functional transcription factor and mediates antiviral activity

WT, Stat1\(^{-/-}\), Stat1\(^{2/-}\) and Stat1ind\(^{BMM}\) cells were pre-treated with dox and stimulated with IFNβ or IFNγ. Dox-treated Stat1ind and WT cells have similar levels of Stat1 mRNA (Fig. 3A, upper panel). The expression levels of Mx1 in Stat1\(^{-/-}\) cells and untreated Stat1ind cells were below detection limits but upon treatment with IFNβ Mx1 was strongly and similarly upregulated in WT and dox-treated Stat1ind cells, whereas it was only weakly induced in Stat1\(^{-/-}\) and dox-untreated Stat1ind cells. As expected, IFNγ-triggered expression of Mx1 was lower than when cells were stimulated with
IFNβ, but was comparable between WT- and STAT1FLAG-expressing cells (Fig. 3A, middle panel). Similar results were found when the expression of Ifi1 was investigated. Only dox-treated Stat1ind cells showed Ifi1 induction upon IFNβ or IFNγ stimulus, with the level of induction comparable to that seen in WT cells (Fig. 3A, lower panel). The data demonstrate the transcriptional activity of STAT1FLAG, which readily reaches WT levels upon IFN treatment.

STAT1 is essential in IFNγ-mediated defense against Vesicular Stomatitis Virus (VSV) [20]. PEFs from WT, Stat1−/− and Stat1ind mice were pretreated with dox, stimulated with serial 2-fold dilutions of IFNγ and either challenged with VSV or left untreated. Cells expressing STAT1FLAG showed similar resistance against the virus to WT cells, while Stat1−/− cells were highly susceptible (Fig. 3B). Consistent with the basal level of STAT1-FLAG expression in Stat1ind PEFs in the absence of dox, these cells showed some antiviral activity, although less than WT. BMMΦs are highly resistant against VSV infections due to their higher levels of endogenous IFN type I [54]. WT and Stat1ind BMMΦs pretreated with dox survived VSV challenge, whereas Stat1ind BMMΦs without dox did not confer any antiviral activity (Fig. S3). STAT1FLAG conferred resistance against VSV, as expected from the IFN-induced expression of ISGs.

In Vivo

To examine the in vivo expression of STAT1FLAG, WT and Stat1ind mice were treated with dox for 3 days and levels of STAT1 protein in various organs analyzed by Western blotting (Fig. 4A and C). In WT mice, the highest amounts of STAT1 were detected in the spleen, with liver and lung also showing high levels. WT STAT1 expression was lower in brain and heart and hardly detectable in kidney and muscle. STAT1FLAG expression was lower compared to WT in brain, similar to that of WT in heart, lung, spleen and muscle, and liver and kidney showed higher levels of STAT1FLAG than of WT STAT1. Analysis of Stat1 mRNA expression revealed that transcription and translation grossly correlate with minor differences observed in heart and spleen (Fig. S5).

Immunohistochemistry was used to obtain more detailed information about the distribution of STAT1FLAG and WT STAT1 in various organs. STAT1FLAG was not detectable in heart, spleen and liver of Stat1ind mice in the absence of dox (Fig. 4B, third row). In skin of Stat1ind mice we detected some mesenchymal, fibroblast-like cells expressing STAT1FLAG also in the absence of dox (Fig. S6, third column). This goes in line with the basal expression of STAT1FLAG observed in PEFs (Fig. S2B). Dox treatment of WT mice had no effect on STAT1 expression (Fig. 4B, first two rows). In spleen and heart, the levels and distribution of STAT1 were similar in WT and dox-treated Stat1ind mice (Fig. 4B, first two columns). In the liver, WT STAT1 was found in Kupffer cells with only no or very low amounts in the hepatocytes, consistent with the notion that different cell types may have different basal levels of STAT1. In dox-treated Stat1ind mice, STAT1FLAG was readily detectable in hepatocytes but not in Kupffer cells (Fig. 4B, right column). Different expression levels of STAT1FLAG compared to WT STAT1 in brain, liver and kidney can be explained by the bioavailability of dox in different cells/organisms and different dox-detoxification rates in vivo. Since Stat1ind expression is not driven by the endogenous Stat1 promoter, the accessibility of the Rosa26 and the Colla1 locus may also contribute to different Stat1ind expression levels in different cell types. However, the results are...
Figure 3. IFN-induced transcription of *Mx1* and *Irf1* at low or WT cellular amounts of STAT1FLAG and antiviral activity of STAT1FLAG.

A) BMMs of various genotypes were pretreated with 0.25 μg/ml dox for 24 h and stimulated with 100 U/ml IFNβ or IFNγ for 4 h or left untreated (w/o). RNA was isolated and cDNA used to analyze expression of Stat1 (upper panel), Mx1 (middle panel) and Irf1 (lower panel) mRNA. Ube2d2 was used for normalization and expression values were calculated relative to untreated WT cells. Results are shown as mean values ± SE from three
consistent with previous reports that the Rosa26 locus drives widespread expression of the tet-activator [46].

**STAT1**<sup>FLAG</sup> expression in *vivo* is dose-dependent and reversible

To examine the dose dependence of STAT1 functions *in vivo*, we applied two different concentrations of dox (0.2 and 1 mg/ml) in the drinking water. The lower dose resulted in lower levels of STAT1<sup>FLAG</sup> in liver, kidney and spleen (Fig. 4C, upper panel). Dox withdrawal resulted in a significant reduction of STAT1<sup>FLAG</sup> after one week (Fig. 4C, lower panel). This proves that STAT1<sup>+</sup> mice express STAT1<sup>FLAG</sup> in a dose-dependent and reversible manner, so they will be useful for examining STAT1 functions that are time-restricted.

**STAT1**<sup>FLAG</sup> reconstitutes MHC-I homeostasis on CD4<sup>+</sup> and CD8<sup>+</sup> splenocytes

Previous studies have shown that homeostatic MHC-I on B and T cells is dependent on STAT1 [56]. To analyze MHC-I expression in splenocytes *in vivo*, we treated WT and STAT1<sup>+</sup> mice with dox for one or two weeks. MHC-I expression was clearly reduced on CD4<sup>+</sup> T cells in STAT1<sup>+</sup> mice without dox (Fig. 4D) as previously shown for STAT1<sup>-/-</sup> mice [56]. Dox treatment for one or two weeks resulted in STAT1<sup>FLAG</sup>-mediated expression of MHC-I on CD4<sup>+</sup> cells and after two weeks of treatment expression was almost at WT levels (Fig. 4D). Similar results were obtained in CD8<sup>+</sup> T-cells (Fig. S7). STAT1<sup>FLAG</sup> is thus able to restore homeostatic MHC-I levels *in vivo*.

**STAT1**<sup>FLAG</sup> confers antiviral activity against VSV and EMCV *in vivo*

STAT1-deficient mice are highly susceptible to VSV, which is mainly cleared through IFN type I activity [19,20,57]. Intravenous (i.v.) challenge with VSV results in high viral loads in spleen and liver [57]. Mice were pretreated with 1 mg/ml dox to give similar or higher STAT1 levels in liver and spleen to those found in WT animals and challenged one week later i.v. with VSV. All WT mice survived, whether or not they were treated with dox. All untreated STAT1<sup>+</sup> mice succumbed to VSV infection within 72 h, as previously shown for STAT1<sup>-/-</sup> mice [19,20] (Fig. 5A, left panel). STAT1<sup>FLAG</sup> expression in mice conferred significantly higher survival upon VSV infection showing that STAT1<sup>FLAG</sup> is functional *in vivo*. However, compared to WT mice the survival was lower. Lowering the dose of dox to 0.2 mg/ml resulted in decreased levels of STAT1<sup>FLAG</sup> in spleen and liver. No significant differences in survival were observed between mice treated with high (Fig. 5A left panel) and low (Fig. 5A, right panel) amounts of dox., i.e. lower levels of STAT1 prior to infection do not impair STAT1-mediated antiviral activity and survival against VSV.

To assess the anti-VSV potency of STAT1<sup>FLAG</sup> in another organ subject to primary infection, we inoculated mice intranasally (i.n.) with VSV. This route of infection leads to infection of the CNS and gives a lethality 3–4 orders of magnitude higher than found with systemic infection [58,59]. VSV challenge via this route resulted in 40% survival of WT mice (compared to 100% after i.v. challenge, Fig. 5A and B). As after i.v. application of the virus, STAT1<sup>+</sup> mice without dox succumbed to the infection within 48 h. Again, reconstitution with STAT1<sup>FLAG</sup> led to improved resistance against VSV. It should be noted that resistance remained lower than in WT animals: the difference does not stem from toxicity of overexpressed hepatic STAT1<sup>FLAG</sup>, as WT and STAT1<sup>+</sup> livers show no pathomorphological differences on day 5 p.i. (Fig. S8A) and high levels of STAT1<sup>FLAG</sup> do not lead to higher expression of ISGs (Fig. S8B).

Finally we tested the resistance to Encephalomyocarditis Virus (EMCV) infection. Intraperitoneal (i.p.) infection with EMCV leads to acute myocarditis and encephalitis and high viral titers in brain, heart, liver and spleen [60,61]. Mice deficient for Ifnar1 or Stat2 are highly susceptible to EMCV, indicating that a functional IFN type I response is essential for host defense [62,63]. We infected dox-pretreated mice i.p. with EMCV (5 pfu/mouse) and monitored survival for 14 days. In the absence of dox, 80% of STAT1<sup>+</sup> mice succumbed to the infection within 48 h, whereas in the presence of dox the survival rate was similar to that of WT mice at about 70% (Fig. 5C). STAT1<sup>FLAG</sup> is thus able to mediate antiviral immunity.

**Discussion**

Here we report the generation of STAT1<sup>+</sup> mice based on a Tet-On [64], i.e. dox-inducible, STAT1 expression system, in which the transgene components are integrated into defined loci that confer sufficient, stable and ubiquitous expression (Fig. 1A and [46]). STAT1<sup>+</sup> mice are homozygous triple mutants, with insertion of the tet activator at the Rosa26 locus, insertion of the dox-dependent FLAG-tagged Stat1 construct at the Colla1 locus and inactivation of the endogenous Stat1 locus. In the uninduced state, STAT1<sup>+</sup> mice are a phenocopy of Stat1<sup>-/-</sup> mice: they are viable and show no obvious abnormalities (Fig. 4 and 5, Stat1<sup>-/-</sup> – dox; [19]). Primary BMMΦs and PEFs show dox dose-dependent inducibility of STAT1<sup>FLAG</sup> to a level clearly exceeding that provided by WT cells (Fig. 1B and Fig. S2B). Biochemical and biological activity of STAT1<sup>FLAG</sup> upon IFN stimulation was assessed by phosphorylation-dependent DNA binding, induction of ISGs and antiviral function and found to be similar to that of WT (Fig. 2 and 3). An interesting aspect of our *in vitro* studies is the observation that BMMΦs expressing very low amounts of STAT1<sup>FLAG</sup> are able to induce ISGs similarly to WT cells upon IFN treatment (Fig. 3C–E). Reconstitution studies of Stat1-deficient cells are usually performed with transient or stable genetic complementation assays using constitutive expression constructs and are not designed to determine the minimal amount of STAT1 required for full biological activity. The Stat1<sup>+</sup> cells provide a suitable tool to study the impact of IFN dose and cell type on ISG expression. In addition they will allow us to address functions of the extranuclear and unphosphorylated STAT1 (U-STAT1) pools: transcriptional complexes containing U-STAT1 are thought to contribute to the
Figure 4. STAT1\(^{\text{FLAG}}\) levels in organs and MHC-I expression on splenocytes. **A, C** WT and Stat1\(^{\text{ind}}\) mice were treated with (i) 1 mg/ml dox in the drinking water for three days (A), (ii) 0.2 mg/ml or 1 mg/ml dox for two weeks (C, upper panel) or (iii) for 1 week with 1 mg/ml dox and in the second week with (+) or without (−) dox (C, lower panel). Brain, heart, liver, lung, spleen, muscle and kidney were isolated and protein extracts used for Western blot analysis. Antibodies against STAT1\(^{a}\) and FLAG were used, with β-tubulin as loading control. **B** WT and Stat1\(^{\text{ind}}\) mice were treated with 1 mg/ml dox in the drinking water for two weeks or left untreated. Heart, spleen and liver were removed and immunohistochemistry performed to analyze STAT1 expression. One representative picture from one of three mice per group is shown. **D** Mice of the indicated genotypes were treated for one or two weeks with 1 mg/ml dox via the drinking water. FACS was used to examine the surface expression levels of MHC-I (H-2Db) on freshly isolated splenocytes. The average level of H-2Db (MFI ± SD) in CD4\(^{+}\) T cells is shown. n = 3 for all genotypes, except for Stat1\(^{\text{ind}}\) without dox after one week treatment (n = 2). P-values between different groups are indicated; p-values above bars indicate significant differences to all other groups compared (* p<0.05; ** p<0.001). doi:10.1371/journal.pone.0086608.g004
delayed phase of the IFN response. U-STAT1 functions can be studied in vitro and in vivo by applying dox in the absence of cytokine (e.g. IFN) and/or by increasing dox dose in the presence of minute amounts of cytokine. With regard to STAT1-isoform specific functions, it has been postulated that the transcriptional inactive STAT1β acts as a dominant negative factor on STAT1α in IFNγ signaling [67]. We did not observe excessive transcriptional or antiviral activity upon IFNγ treatment in cells expressing STAT1α only.

In vivo dox-induced STAT1FLAG was detected in all organs analyzed (Fig. 4). We could not detect transgenic STAT1 in the absence of dox in cells (apart from fibroblasts), tissues or whole organs (apart from heart), confirming the tightness of the system [46,47]. Dox-induced expression of STAT1FLAG in vivo resulted in levels of STAT1 that were similar, higher or lower to those seen in the WT, depending on the tissue/organ analyzed and the dose of dox administered. STAT1FLAG expression was not always comparable to that of WT-STAT1 in all organs – most likely a result of different accessibility of the Col1a1 and the Rosa26 locus and varying disposability of dox in different organs. Since COL1A1 is highly expressed in fibroblasts, we speculate that the observed leakiness of the system in this cell type is due to residual transcription of the Stat1ind construct from the Col1a1 locus [55,68]. While the recently described conditional Stat1 mice [28,50] allow the irreversible manipulation of STAT1 expression, Stat1ind mice facilitate time-restricted STAT1FLAG expression by simple depriorituation of dox. Nevertheless, it should be noted that, depending on the tissues or organs studied, dox deprivation requires periods of up to 4 weeks and even thereafter low-level release from depots has been reported [69,70]. Tissue-specific production of STAT1-FLAG can be readily achieved by replacing the Rosa26-controlled tet transactivator by cross-breeding with one of the various tissue-/cell-type-specific rtTA mice [71].

Survival upon challenge with viruses revealed that levels of STAT1FLAG suffice to confer WT response to EMCV, while upon VSV infection mice expressing dox-induced STAT1 FLAG performed significantly better than untreated controls, although their survival rates did not reach WT levels (Fig. 5). In experimental settings the two viruses have different primary target organs and pathogenic mechanisms as well as differing molecular interfaces with the host immune system [60,72–74]. To clear the infection in each case the host depends on a functional IFN-I response, and hence on STAT1, although the temporal and spatial STAT1 activities required for host survival are unknown.

The reasons for the failure of STAT1 FLAG to clear VSV infection completely under our experimental conditions most likely relate to inappropriate levels of STAT1 in particular organs and tissues and/or to unbalanced STAT1 levels in a given cell type. As mentioned above, in view of the pharmacodynamics of dox after oral application [75,76] and of the variations in the chromatin accessibility of the transgene-controlling loci between various tissues and stages, it is not to be expected that STAT1FLAG levels

Figure 5. STAT1FLAG-mediated defense against VSV and EMCV infections. WT and Stat1ind mice were pretreated with the indicated concentrations of dox in the drinking water for one week or left untreated A) Mice were injected with 10⁵ pfu/mouse VSV intravenously (i.v.) and survival monitored for two weeks. Numbers of mice are indicated and results are shown from four (left panel) or two (right panel) independent experiments. There was no significant difference in survival between WT mice treated with or without dox. The survival of Stat1ind mice with or without dox was significantly different (p<0.0002) from that of all other groups. B) Mice were injected with 10⁵ pfu/mouse VSV intranasally (i.n.) and survival monitored for two weeks. Results are shown from three independent experiments. The survival rate of Stat1ind mice without dox and Stat1ind mice with dox was significantly different (p<0.0001) from that of all other groups. C) Mice were injected with 5 pfu/mouse EMCV intraperitoneally (i.p.) and survival monitored for two weeks. Results are shown from two independent experiments. The survival of Stat1ind mice without dox was significantly different (p<0.0024) from that of all other groups.

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at a given dose and route of application of dox will match the levels of WT STAT1 in all organs and tissues [77]. It should be noted that STAT1FLAG is massively overexpressed in hepatocytes but, in contrast to WT, not detectable in Kupffer cells. Hepatic STAT1 expression is not toxic, as we did not see any obvious changes in immunohistochemistry (Fig. S8A). Tests of the hepatic expression of ISGs showed similar amounts of Mx1 and Ifi27 mRNA in mice expressing WT STAT1 and STAT1FLAG after 5 days of VSV infection. Notably, overexpressed STAT1FLAG did not translate to higher levels of ISGs (Fig. S8B). In addition, reconstitution with STAT1FLAG led after two weeks to restoration of WT MHC-I expression on splenocytes. It is conceivable that restoring the chromatin landscape of the MHC-I locus and probably also of other important loci for innate and adaptive immunity might be more complex and time-consuming than restoring STAT1 levels [16].

The importance of balanced cellular STAT levels has been shown for infection with Lymphocytic Choriomeningitis Virus (LCMV): the ratios of basal and induced STAT1/STAT4 determine the innate and adaptive immune cell activities [14,15]. Imbalances in the cellular STAT ratio in dox-treated Stat1ind mice might therefore contribute to the incomplete defense against VSV. Future studies will address these issues and determine the dose, application route and duration of dox treatment required to generate appropriate levels of STAT1 in organs/cell types of interest.

In summary, we report the generation of the first functional, regulatable and reversible Stat1 transgenic mouse that enables the study of STAT1 functions under conditions of under- and overexpression or of normal expression, in the phosphorylated or unphosphorylated state. The Stat1ind mice will serve as a powerful tool to study the contribution of STAT1 to the onset, progression and outcome of disease.

Supporting Information

Figure S1 Southern Blot analysis of ES cells targeted with a dox-inducible Stat1 construct. A) Schematic organization of the Col1a1 locus in WT (upper panel), KH2 (middle panel) and Stat1ind cells (lower panel). Open boxes represent the last exons of the Col1a1 gene. SpeI restriction sites, the probe used for Southern blot analysis and resulting fragments are indicated. pA: polyadenylation signal; frt: flippase recognition target; P: promoter; neo: neomycin resistance cassette; hygro: hygromycin resistance cassette; SA: splice acceptor; tetO: tetracycline operator. B) DNA was isolated from WT, KH2 and Stat1ind ES cells and digested with SpeI and Southern blotting was performed using a probe against the Col1a1 locus. Fragments of 6.2 kb refer to WT DNA; KH2 cells harboring frt sites result in fragments of 6.7 kb (frt); and Stat1ind DNA gives rise to a 4.1 kb fragment (frp-in). (TIF)

Figure S2 Dox time- and dose-dependent expression of STAT1FLAG in ES cells, PEFs and splenocytes. A) Stat1ind ES cells were treated with 0.1, 1 or 10 μg/ml dox for 24 h or left untreated. PEFs (B) and splenocytes (C) were isolated from Stat1ind mice and dox-treated with different amounts for 24 h (upper panels) or for different times (lower panels). A, B, C) Western blot was performed to analyze the expression of STAT1, membranes were reprobed with a FLAG-specific antibody. Loading was controlled with panERK or β-tubulin. Splenocytes were isolated from whole spleens mashed through a 100 μm cell strainer and red blood cells removed using Red Blood Cell Lysis Buffer (Sigma). Splenocytes were grown for 5 days in RPMI medium supplemented with 10% FCS, 2 mM L-Glutamin, Penicillin/Streptomycin (100 μg/ml and 100 U/ml), 50 μM β-Mercaptoethanol and 2 μg/ml Concanavalin A (all Sigma). (TIF)

Figure S3 Antiviral activity of STAT1FLAG in BMMΦs. BMMΦs were isolated from WT and Stat1ind mice and 4×10^5 cells plated onto each well of a 96-well plate. Cells were treated with 0.25 μg/ml dox for 48 h and subsequently infected with serial 2-fold dilutions of VSV starting at a moi of 100. After 40 h surviving cells were stained with crystal violet. (TIF)

Figure S4 Dox dose-dependent expression of STAT1FLAG in BMMΦs. BMMΦs were isolated from WT and Stat1ind mice and stimulated with indicated amounts of dox for 24 h or left untreated. Protein lysates were used to perform WB to analyze STAT1 and STAT1FLAG expression, panERK was used as loading control. One representative blot from three independent experiments is shown. (TIF)

Figure S5 Stat1ind expression in organs. WT and Stat1ind mice were treated with 1 mg/ml dox in the drinking water for three days. RNA was isolated from brain, heart, liver, lung, spleen, muscle and kidney and cDNA used to analyze Stat1 expression. Ube2d2 was used for normalization and expression values were calculated relative to each untreated WT organ. Results are shown as mean values ± SE from three animals per genotype and treatment from two independent experiments. P-values above bars indicate significant differences compared to all other groups (⁎ p<0.05; ⁎⁎p<0.01; ⁎⁎⁎ p<0.001). (TIF)

Figure S6 STAT1FLAG expression in skin. WT and Stat1ind mice were treated with 1 mg/ml dox in the drinking water for three days or left untreated. Skin was removed and immunohistochemistry performed to analyze STAT1 expression. One representative picture from one of two mice per group is shown. (TIF)

Figure S7 MHC-I expression on CD8+ splenocytes. Mice of the indicated genotypes were treated for one or two weeks with 1 mg/ml dox via the drinking water. FACS was used to examine the surface expression levels of MHC-I (H-2Db) on freshly isolated splenocytes. The average level of H-2Db (MFI±SD) in CD8+ T cells is shown. n = 3 for all genotypes, except for Stat1ind Stat1ind without dox after one week treatment (n = 2). P-values between different groups are reported; p-values above bars indicate significant differences to all other groups (⁎ p<0.05; ⁎⁎p<0.01; ⁎⁎⁎ p<0.001). (TIF)

Figure S8 Expression of Stat1 protein and Stat1, Mx1 and Ifi27 mRNA in liver of VSV-infected mice. WT and Stat1ind mice were treated with 1 mg/ml dox for one week and subsequently injected i.v. with VSV (10^7 pfu/mouse; +VSV) or as a control with PBS (−VSV). A) Liver was isolated on day 5 p.i. to analyze STAT1 expression by immunohistochemistry. One representative picture from one out of three mice per group is shown. B) Total RNA was isolated from liver 5 days p.i. and cDNA was used to analyze expression of Stat1 (upper panel), Mx1 (middle panel) and Ifi27 (lower panel) mRNA. Values were normalized to Ube2d2 and calculated relative to PBS-treated WT mice. Results are shown as mean values ± SE; p-values (⁎⁎⁎ p<0.001) are indicated. n = 2 for PBS treated mice, n = 3 for VSV-treated mice. (TIF)
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Author Contributions

Conceived and designed the experiments: MM NRL. Performed the experiments: NRL CR RL SH BB LK. Assisted reagents/materials/analysis tools: RE SM TK TR. Wrote the paper: NRL MM BS.
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