The methyltransferase Setdb2 mediates virus-induced susceptibility to bacterial superinfection

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Immune responses are tightly regulated to ensure efficient pathogen clearance while avoiding tissue damage. Here we report that Setdb2 was the only protein lysine methyltransferase induced during infection with influenza virus. Setdb2 expression depended on signaling via type I interferons, and Setdb2 repressed expression of the gene encoding the neutrophil attractant CXCL1 and other genes that are targets of the transcription factor NF-κB. This coincided with occupancy by Setdb2 at the Cxcl1 promoter, which in the absence of Setdb2 displayed diminished trimethylation of histone H3 Lys9 (H3K9me3). Mice with a hypomorphic gene-trap construct of Setdb2 exhibited increased infiltration of neutrophils during sterile lung inflammation and were less sensitive to bacterial superinfection after infection with influenza virus. This suggested that a Setdb2-mediated regulatory crosstalk between the type I interferons and NF-κB pathways represents an important mechanism for virus-induced susceptibility to bacterial superinfection.

Secondary bacterial pneumonia has a predominant role in the morbidity of seasonal and pandemic infection with influenza virus and thus represents a considerable clinical as well as socioeconomic challenge. Virus-induced immune responses are thought to be involved in the pathogenesis of bacterial superinfection, yet the molecular mechanisms of this remain poorly understood. The recognition of pathogens by receptors such as Toll-like receptors (TLRs) leads to the induction of two major signaling pathways: that of type I interferons, and that of the transcription factor NF-κB. The transcription of type I interferons is regulated by the family of interferon–regulatory factors (IRFs). Secreted interferons bind to the ubiquitously expressed heteromeric receptor for interferon-α (IFN-α) and IFN-β, composed of IFNAR1 and IFNAR2, which results in the expression of a large number of interferon-stimulated genes (ISGs). Many ISGs encode effector proteins that mediate defense against viruses and other pathogens. The same triggering of TLRs can lead to the activation of NF-κB proteins and their translocation to the nucleus, which in turn induces the expression of genes encoding proinflammatory molecules involved in antibacterial defense. Signaling via type I interferon and NF-κB is subjected to multiple layers of regulation that are needed to maintain a balance among effective pathogen clearance, the prevention of tissue damage and disease tolerance. This is of particular relevance for superinfections, during which virus-induced host responses can lead to increased susceptibility to bacterial infections through type I interferon–mediated interference with NF-κB signaling. Immune responses are shaped by chromatin modifications.

Here we have identified and functionally characterized the protein lysine methyltransferase (PKMT) Setdb2 (‘suvar 3-9–enhancer-of-zeste–trithorax (SET) domain bifurcated 2’; UniProt accession code Q8C267) as an interferon-stimulated protein that modulates the expression of a subset of genes that are targets of NF-κB. Setdb2 belongs to the SUV39 family, whose members share a SET domain related to Setdb2, is involved in proviral silencing, genomic stability and the onset of cancer. So far, Setdb2 has been linked functionally to embryonic development and cell division. In this study we report a role for Setdb2 as a critical interferon-stimulated regulator of...
the immune system that contributes to the molecular mechanisms of virus-induced susceptibility to bacterial superinfection.

RESULTS

Induction of Setdb2 expression by infection with influenza virus

To identify previously unknown regulatory immunological mechanisms involved in virus-induced susceptibility to bacterial superinfection, we infected wild-type mice with influenza virus and collected lung tissue at 18 h after infection. At this early time point, the distribution of viral antigen was limited to a small percentage of epithelial cells (Fig. 1a). We next performed global expression profiling of lung tissue from infected and uninfected wild-type mice. We identified more than 200 virus-induced genes, many of which were known ISGs (Supplementary Table 1). Gene-ontology analysis highlighted enrichment of this group for genes encoding molecules involved in interferon-mediated immune responses, which included the gene-ontology terms ‘interferon alpha and interferon beta signaling’ (standard name (Broad Institute), Reactome; systematic name (Broad Institute)), M973; P = 0.0 × 10^10) and ‘interferon-mediated immunity’ (PANTHER; systematic name, BP00156; P = 5.00 × 10^11), IRF8 (P = 0 × 10^0), IRF7 (P = 1.70 × 10^−8), ‘macrophage-mediated immunity’ (PANTHER; systematic name, BD00155; P = 5.00 × 10^12) and ‘cytokine/chemokine-mediated immunity’ (PANTHER; systematic name, BP00155; P = 0 × 10^0), ‘chemokine signaling pathway’ (Kyoto Encyclopedia of Genes and Genomes; systematic name, M4844; P = 4.85 × 10^−14), ‘Toll-like receptor signaling pathway’ (Kyoto Encyclopedia of Genes and Genomes; systematic name, M3261; P = 7.89 × 10^−8) and ‘inflammation’ (Kyoto Encyclopedia of Genes and Genomes; systematic name, M06100; P = 6.10 × 10^−11).

Next we analyzed the expression of the mouse orthologs of previously annotated PKMTs. This revealed Setdb2 as the only PKMT with significant induction of expression upon infection with influenza virus (Fig. 1b and Supplementary Table 1).

Setdb2 expression driven by type I interferon signaling

We identified several putative IRF-binding sites by motif scanning of Setdb2 (Supplementary Table 2). To investigate whether the

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**Figure 1** Setdb2 expression is induced in an IFNAR1-dependent manner upon infection with influenza virus and stimulation of TLRs. (a) Microscopy of sections of lungs obtained from wild-type mice left uninfected (UI) or assessed 18 h after intranasal infection with influenza virus PR8 (left margin), then stained with an antibody specific for influenza virus H1N1 (Anti-H1N1); arrowheads indicate influenza virus H1N1–infected areas. Scale bar, 250 μm. (b) Expression of genes encoding PKMTs (right margin) in lungs of mice after infection with influenza virus PR8 relative to their expression in lungs of mice left uninfected (mean value (log2); left margin), and robust multi-array average (RMA) values for expression in individual mice (columns in middle). (c) Real-time PCR analysis of Setdb2 expression in lung tissue from wild-type, Irf7−/−, Irf8−/− and Stat1−/− mice left uninfected or 18 h after intranasal infection with influenza virus PR8 (key); results are presented relative to those of uninfected wild-type mice, set as 1. Each symbol represents an individual mouse; small horizontal lines indicate the mean. (d–f) Real-time PCR analysis of Setdb2 expression (top) and immunoblot analysis of Setdb2 (detected with monoclonal antibody 7H7F11), Zbp1 (induction control) and actin (loading control throughout) (bottom) in BMDCs from Irfar−/− and wild-type mice (key) left uninfected or infected with influenza virus PR8 (multiplicity of infection, 10) (d) or left unstimulated (US) or stimulated with IFN-β, IFN-γ or IFN-λ (e) or TLR agonists Pam3CSK4 (PAM3), poly(I:C) or LPS (f), assessed 8 h after treatment (mRNA) or 8 h (d) or 24 h (e, f) after treatment (protein); mRNA results are presented relative to those of untreated wild-type cells, set as 1. Right margin (bottom), size markers in kilodaltons (kDa). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 (unpaired t-test). Data are from one experiment with three mice per condition (a) or one experiment representative of two independent experiments with similar results (d–f; mean and s.e.m. of biological triplicates) or are pooled from two independent experiments with six mice per condition (b) or two independent experiments (c).
induction of Setdb2 depended on interferon signaling, we infected wild-type mice as well as IFNAR1-deficient (Ifnar1−/−) mice, IRF7-deficient (Irf7−/−) mice and mice deficient in the type I interferon effector STAT1 (Stat1−/− mice), each of which lack key molecules required for interferon signaling, with influenza virus. We collected lung tissue at 18 h after infection and detected higher expression of Setdb2 in the lungs of infected wild-type mice than in those of uninfected wild-type mice (Fig. 1c). In contrast, the induction of Setdb2 was much lower in the lungs of infected Ifnar1−/− mice than in the lungs of infected wild-type mice (Fig. 1c), which indicated that type I interferon signaling was essential for the upregulation of Setdb2 in vivo. We also observed lower Setdb2 expression in lungs from infected Irf7−/− and Stat1−/− mice than in the lungs of infected wild-type mice (Fig. 1c). Similarly, infection of primary mouse bone marrow–derived macrophages (BMDMs) with influenza virus resulted in IFNAR1-dependent upregulation of Setdb2 transcription (Fig. 1d). To investigate protein expression, we raised a monoclonal antibody (clone 717F11) against the carboxy-terminal region of mouse Setdb2. Consistent with the expression of mRNA encoding Setdb2, we detected increased Setdb2 protein, in an IFNAR1-dependent manner, after infection of BMDMs (Fig. 1d). Detection of the known interferon-stimulated protein Zbp1 (DAI) served as a control. Stimulation of BMDMs with IFN-β (type I interferon), IFN-γ (type II interferon) or IFN-λ (type III interferon) revealed that Setdb2 and Zbp1 were induced by the type I interferon as well as by the type II interferon but not the type III interferon (Fig. 1e). The induction of Setdb2 by the type II interferon was partially dependent on IFNAR1, which indicated a secondary requirement for endogenous type I interferon27.

Next we elucidated the expression profiles of Setdb2 mRNA and Setdb2 protein after stimulation of BMDMs with the synthetic lipopeptide and TLR2 agonist Pam 3CSK4, the synthetic viral RNA analog and TLR3 agonist polyinosinic-polycytidylic acid (poly(I:C)) or the TLR4 agonist lipopolysaccharide (LPS)5. Treatment of wild-type BMDMs in this way resulted in upregulation of both Setdb2 mRNA and Setdb2 protein (Fig. 1f). Again, this induction was entirely dependent on type I interferon signaling, as Setdb2 was not induced in Ifnar1−/− BMDMs (Fig. 1f). The induction of Setdb2 and Zbp1 upon stimulation with Pam3CSK4 was TLR2 dependent (Supplementary Fig. 1). In addition to inducing Setdb2, stimulation of wild-type BMDMs with poly(I:C) led to the induction of genes encoding other PKMTs, such as Setd1b and Prdm1, which encodes Blimp-1, a well-studied regulator of B cells and T cells; however, expression of these genes was not altered upon infection with influenza virus in vivo (Fig. 1b, Supplementary Fig. 2 and Supplementary Table 3).
Figure 3 Setdb2 binds to the Cxcl1 promoter region and associates with H3K9me3. ChIP analysis of endogenous Setdb2 (with 7H7F11) (a), H3K9me3 (b) or H3K9ac (c) or empty beads (mock (M)) in wild-type or Setdb2<sup>GT/GT</sup> BMDMs left unstimulated (US; b only) or stimulated for 2 h with poly(I:C), followed by real-time PCR analysis for specific enrichment of each at the Cxcl1 promoter (prom) or exon 1 (ex1) or the control gene Actb (which encodes β-actin); results were normalized to the highest recovery value in wild-type cells. *P ≤ 0.05, **P ≤ 0.001 and ***P ≤ 0.0001 (unpaired t-test). Data are from two independent experiments (cells, a,b) or one experiment (empty beads, a,b) or one experiment (c) with duplicate measurements (mean and s.e.m.).

The elevated expression of Setdb2 mRNA in wild-type BMDMs persisted for at least 24 h (Supplementary Fig. 2). Together these data indicated that interferon signaling was required for the induction of Setdb2 both during viral infection and upon stimulation of TLRs.

Modulation of expression of NF-κB target genes by Setdb2

We generated mice with a hypomorphic gene-trap construct of Setdb2 (Setdb2<sup>GT/GT</sup> mice) to investigate the biological function of Setdb2 (Supplementary Fig. 3). Immunoblot analysis of spleen, lungs and BMDMs showed much less Setdb2 protein in Setdb2<sup>GT/GT</sup> mice than in wild-type mice (Fig. 2a). Next we compared the transcriptomes of untreated and poly(I:C)-stimulated wild-type and Setdb2<sup>GT/GT</sup> BMDMs by high-throughput sequencing technology for cDNA (RNA-Seq). This revealed significant enrichment for known NF-κB target genes among the transcripts upregulated in Setdb2<sup>GT/GT</sup> BMDMs (P = 1.35 × 10<sup>−8</sup> (hypergeometric test); Fig. 2b and Supplementary Tables 4 and 5). These NF-κB target genes represented a subset of genes that included Cxcl1, Il12b, S100a8, S100a9, Lcn2, Cxcl2, Chi3l1 and Ltf, each of which encodes a product linked to antibacterial defense. However, other established NF-κB-regulated genes, such as Il6 (which encodes the proinflammatory cytokine interleukin 6 (IL-6)), were not affected (Supplementary Table 4). We found no differences between stimulated wild-type BMDMs and stimulated Setdb2<sup>GT/GT</sup> BMDMs in their upstream activation of the NF-κB cascade, as measured by degradation of the NF-κB inhibitor IκBα (Supplementary Fig. 4), which suggested that Setdb2 acted downstream of IκBα-mediated activation of NF-κB.

One of the genes found to have particularly high expression in poly(I:C)-stimulated Setdb2<sup>GT/GT</sup> BMDMs was Cxcl1. This encodes a key chemoattractant for neutrophils, a type of leukocyte shown to be critically involved in bacterial clearance during superinfection<sup>13–15</sup>. Thus, we decided to focus on the effects of Setdb2-mediated regulation of Cxcl1. Indeed, stimulation with various TLR agonists resulted in significantly more induction of Cxcl1 transcripts in Setdb2<sup>GT/GT</sup> BMDMs than in wild-type control BMDMs (Fig. 2c). We confirmed that finding at the level of protein secreted by BMDMs (Fig. 2d). To investigate the effects of type 1 interferon signaling on Cxcl1 expression, we stimulated BMDMs with poly(I:C) in the presence of IFNAR1-blocking antibodies. This led to diminished levels of Setdb2 mRNA, which correlated inversely with the expression of Cxcl1 mRNA (Supplementary Fig. 5). We saw these differences not only in wild-type BMDMs but also in Setdb2<sup>GT/GT</sup> BMDMs; this may have been due to the residual levels of Setdb2 in the Setdb2<sup>GT/GT</sup> BMDMs and/or another type I interferon–driven Setdb2-independent pathway. Finally, infection with influenza virus strain PR8 led to higher expression of Cxcl1 and a higher concentration of CXCL1 in Setdb2<sup>GT/GT</sup> BMDMs than in wild-type BMDMs (Fig. 2e,f). These data suggested that Setdb2 was a negative regulator of a subset of genes that are targets of NF-κB.

Setdb2 mediates trimethylation of H3K9 at the Cxcl1 promoter

Methyltransferases of the SUV39 family ‘preferentially’ methylate the histone substrate H3K9 (refs. 20,28). Setdb2 has been shown to catalyze the addition of the repressive mark trimethylated H3K9 (H3K9me3)<sup>23,24</sup>. We therefore hypothesized that Setdb2 would inhibit Cxcl1 expression by introducing repressive marks in the Cxcl1 promoter region. To determine if Setdb2 was able to bind to the Cxcl1 promoter, we used the Setdb2-specific monoclonal antibody 7H7F11 for chromatin immunoprecipitation (ChIP). We treated BMDMs for 2 h with poly(I:C) and quantified Setdb2-specific enrichment of genomic DNA by real-time PCR. In these ChIP assays we found a significant enrichment in the binding of Setdb2 to the promoter region of Cxcl1 (Fig. 4).
region and exon 1 of Cxcl1 compared with its binding to the promoter sequence of the constitutively expressed control gene Actb (Fig. 3a). This indicated that Setdb2 bound to the Cxcl1 promoter.

Next we addressed whether Setdb2 binding correlated with altered chromatin modifications. For this, we performed ChIP analysis of H3K9me3 in the presence or absence of poly(I:C) stimulation of wild-type or Setdb2<sup>GT/GT</sup> BMDMs. Under unstimulated conditions, Setdb2<sup>GT/GT</sup> cells had less H3K9me3 at the promoter and exon 1 of Cxcl1 than did wild-type cells (Fig. 3b). This repressive mark was significantly increased at the Cxcl1 promoter region upon stimulation of wild-type cells with poly(I:C) (Fig. 3b), consistent with the rapid induction but only transient expression of this gene (Supplementary Fig. 6). Such an increase in H3K9me3 was absent from Setdb2<sup>GT/GT</sup> cells (Fig. 3b). The promoter of the actively transcribed gene Actb had a low degree of trimethylation of H3K9 in both wild-type BMDMs and Setdb2<sup>GT/GT</sup> BMDMs (Fig. 3b). As a control, we analyzed the presence of the activation mark of acetylated H3K9 (H3K9ac) and, as expected, found enrichment for H3K9ac at the promoter of Actb (Fig. 3c). Together these data suggested that Setdb2 mediated the repression of Cxcl1 expression at the chromatin level.

Setdb2 deficiency results in exacerbated lung inflammation
Our experiments with Setdb2<sup>GT/GT</sup> BMDMs revealed increased expression of CXCL1, which as a chemoattractant for neutrophils is important for efficient pathogen clearance as well as implicated in immunopathologies<sup>28</sup>. To confirm our findings, we challenged wild-type and Setdb2<sup>GT/GT</sup> mice with a model of LPS-induced pulmonary neutrophilia. At 4 h after an intranasal application of LPS, we...
obtained bronchoalveolar lavage (BAL) fluid to assess potential changes in CXCL1 secretion and cell infiltration. In addition, we extracted total RNA from lung tissue to analyze differences in gene expression. This demonstrated higher expression of Cxcl1 mRNA (Fig. 4a) and more secretion of CXCL1 protein (Fig. 4b) in Setdb2\textsuperscript{GT/GT} mice than in wild-type mice. The increase in CXCL1 was accompanied by elevated total cell infiltration (Fig. 4c), as well as neutrophilia in the airways, as measured by cytospin (Fig. 4d). Macrophage numbers were similar in wild-type mice and Setdb2\textsuperscript{GT/GT} mice (Fig. 4e). We concluded that Setdb2\textsuperscript{GT/GT} mice showed increased infiltration of neutrophils into the lungs in the early phase of inflammation.

**Setdb2 mediates susceptibility to bacterial superinfection**

The rapid recruitment of neutrophils by the early cytokine CXCL1 is crucial for the prevention of excessive lung inflammation as well as for the clearance of *Streptococcus pneumoniae*, the most common bacterial agent found in superinfection after primary infection with influenza virus\textsuperscript{1,14,15,30}. Therefore, we sought to investigate the response of Setdb2\textsuperscript{GT/GT} mice in a superinfection model of *S. pneumoniae* after primary infection with influenza virus.

Upon intranasal infection with influenza virus, Setdb2\textsuperscript{GT/GT} mice underwent more induction of CXCL1 than did wild-type mice (Fig. 5a,b). In the lungs, CXCL1 is secreted by various cell types, including alveolar macrophages\textsuperscript{31}. Indeed, we obtained alveolar macrophages ex vivo from BAL fluid and found that after infection of the cells with influenza virus, Setdb2\textsuperscript{GT/GT} cells had higher expression of CXCL1 than did cells derived from wild-type mice (Supplementary Fig. 7), which suggested this cell population as one source of CXCL1 in our infection model *in vivo*. The expression of other inflammatory mediators, such as CXCL2, IL-6 and IL-10, was similar in BAL fluid from Setdb2\textsuperscript{GT/GT} mice and that from wild-type mice (Supplementary Fig. 8a–c). Despite the increased amount of CXCL1 in Setdb2\textsuperscript{GT/GT} mice upon infection with influenza virus, both mouse strains showed similar infiltration of neutrophils into the lungs at this stage of infection (Fig. 5c,d). Comprehensive profiling of other cell populations in the lung tissue and BAL fluid, including monocytes, macrophages, dendritic cells, alveolar macrophages, natural killer cells, T cells and B cells, revealed no genotype-specific differences in uninfected and influenza virus–infected Setdb2\textsuperscript{GT/GT} and wild-type mice (Supplementary Fig. 9a–d). The viral load was similar in both mouse strains (Supplementary Fig. 10a). After single infection with *S. pneumoniae*, Setdb2\textsuperscript{GT/GT} and wild-type mice exhibited no substantial difference in the induction of CXCL1 (Supplementary Fig. 8d), the number of neutrophils (Supplementary Fig. 9e,f) or the bacterial burden (Supplementary Fig. 10b).

Superinfection of influenza virus–infected mice with *S. pneumoniae* led to a further increase in CXCL1 (Fig. 5b). This was accompanied by increased infiltration of neutrophils into lung tissue and in the BAL fluid of Setdb2\textsuperscript{GT/GT} mice but not that of wild-type mice (Fig. 5c,d, Supplementary Fig. 9g,h). At 2 d after superinfection with *S. pneumoniae*, mice showed severe signs of pneumonia (Fig. 5e) and pulmonary edema, as measured by lung wet weight (Fig. 5f). The gross pathological appearance, including size, weight, number of affected lobes and hemorrhagic lesions, was milder in Setdb2\textsuperscript{GT/GT} lungs than in wild-type lungs (Fig. 5e,f). Histopathological analysis of lung sections confirmed and extended the macroscopic findings, with diminished signs of pneumonia, including bronchitis, endothelialitis and inflammatory infiltrates, in Setdb2\textsuperscript{GT/GT} lungs compared with that in wild-type lungs (Fig. 5g,h). In line with the ameliorated pathological findings in Setdb2\textsuperscript{GT/GT} mice at this advanced stage of bacterial superinfection, we found less Il6 mRNA and IL-6 protein in Setdb2\textsuperscript{GT/GT} mice than in wild-type mice (Fig. 5i,j) and a lower bacterial burden in Setdb2\textsuperscript{GT/GT} mice than in wild-type control mice (Fig. 5k). Together these data indicated that influenza virus–induced expression of Setdb2 had a negative effect on the early recruitment of neutrophils, pathogen clearance and tissue integrity during bacterial superinfection.

**DISCUSSION**

Maintaining a balance between effective pathogen defense and the prevention of excessive inflammation, autoimmunity and immunopathology is the central task of immunoregulation\textsuperscript{10,12}. Signaling via type I interferons and signaling via NF-κB are two important pathways for this process and are subjected to multiple layers of crosstalk, many of which are still poorly understood. In particular, chromatin modifiers are being increasingly recognized as crucial mediators and effectors of immunoregulatory mechanisms.

Here we identified Setdb2 as part of the interferon-mediated immune response that provides a previously unknown layer of regulatory crosstalk between the signaling pathways of type I interferon and NF-κB. The Setdb2–related SUV39 family members Suv39H1, Ezh1 (Glp) and Ezh2 (G9a) are involved in immunological processes such as the modulation of ISG expression, the NF-κB pathway and T cell differentiation\textsuperscript{17,19,32}. Our finding that Setdb2 was itself an ISG indicated a role for Setdb2 in the innate immune response, which might contribute to preventing excessive immunopathology—in particular, infections and/or inflammatory conditions. Notably, single-nucleotide polymorphisms in human SETDB2 have been shown to be associated with increased production of immunoglobulin E and atopic asthma\textsuperscript{33}, which supports the proposed role of Setdb2 in regulating inflammation. This evolutionary strategy, however, may turn into a double-edged sword during bacterial superinfection by causing impaired bacterial clearance and severe tissue damage. Signaling via type I interferon has been shown to have a detrimental role in the pathogenesis of virus–induced susceptibility to bacterial superinfection\textsuperscript{4,13–15}. Accordingly, Setdb2 may be responsible for and mediate at least part of this type I interferon–dependent mechanism.

Chromatin modifiers can be recruited to their targets through specific interactions with transcriptional regulators and/or chromatin-associated factors. We hypothesize that a similar mechanism facilitates the specific recruitment of Setdb2 to the promoters of its target genes to introduce repressive H3K9me3 chromatin marks. However, we cannot exclude the possibility that Setdb2 may recruit other methyltransferases or transcription factors. Likewise, the function of Setdb2 may be determined by non–mutually exclusive cellular and immunological parameters (for example, cell type, inflammatory state and pathogen type) as well as by the complex epigenetic context of multivalent chromatin modifications\textsuperscript{34}. Independently of direct histone methylation and in analogy to other PKMTs, Setdb2 may also methylate non-histone protein targets\textsuperscript{32,35}.

Our study has shown that reduced amounts of Setdb2 led to increased production and secretion of CXCL1. Our data suggested that the increased CXCL1 expression and neutrophil recruitment observed in Setdb2\textsuperscript{GT/GT} mice might have been causally involved in the ameliorated pathogenesis of bacterial superinfection. Neutrophils serve various roles, including the regulation and resolution of inflammation and the elimination of bacterial pathogens\textsuperscript{29,36}. In this study, we observed higher CXCL1 expression in the lungs of influenza virus–infected Setdb2\textsuperscript{GT/GT} mice than in those of their wild-type counterparts. This alteration in chemokine production and secretion was not sufficient to augment the virus–induced neutrophil response. The increased CXCL1 expression in Setdb2\textsuperscript{GT/GT} mice manifested phenotypically
only upon superinfection as elevated numbers of neutrophils in the lungs. This could have been due to altered time kinetics of the host response and/or the involvement of additional signals provided by the bacterial superinfection. Together our data suggested that the influenza virus–induced expression of Setdb2 reduced the antibacterial response in wild-type mice, leading to aggravated lung pathology.

Our findings have provided evidence of a regulatory function for Setdb2 in CXCL1 expression, the recruitment of neutrophils, and the pathological outcome of superinfection. However, we cannot exclude the possibility that the products of other Setdb2-modulated genes might have contributed to the improved bacterial clearance and diminished tissue inflammation in Setdb2<sup>GT/GT</sup> mice. Our RNA-Seq experiment with poly(I:C) stimulation identified several other genes encoding antibacterial molecules that were upregulated in Setdb2<sup>GT/GT</sup> BMDMs. These included genes encoding the chemotactic proteins S100a8 and S100a9 (ref. 37); Marco, a macropage scavenger receptor linked to the phagocytosis and clearance of bacteria in the lungs<sup>38</sup>; and Chi3l1, which has been shown to promote both improved clearance of <i>S. pneumoniae</i> and disease tolerance<sup>39</sup>. However, expression profiling of these genes by real-time PCR revealed no difference between superinfected wild-type mice and superinfected Setdb2<sup>GT/GT</sup> mice under the experimental conditions of this study (data not shown). Finally, Setdb2 may not only regulate antibacterial responses but also modulate genes encoding molecules involved in disease tolerance in superinfection<sup>40</sup>.

In summary, our study has assigned to Setdb2 a regulatory role in the interferon-mediated immune response and in the pathogenesis of virus-induced susceptibility to bacterial superinfection. Several inhibitors for PKMTs are currently in clinical trials<sup>41</sup>. Thus, Setdb2 could be a therapeutic target for the treatment of superinfections and other inflammatory conditions.

METHODS

Methods and any associated references are available in the online version of the paper.

Accessory codes. ArrayExpress: microarray data, E-MTAB-2845 and E-MTAB-2263.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.S. designed experiments, performed in vitro and in vivo studies and wrote the manuscript; E.K.F., A.L.T., L.T. and D.E.S. generated the Setdb2<sup>GT/GT</sup> mouse and provided advice; B.V. performed and provided flow cytometry and Chip-DNA experiments; Y. Guo performed real-time PCR revealed no difference between superinfected wild-type mice and superinfected Setdb2<sup>GT/GT</sup> mice under the experimental conditions of this study (data not shown). Finally, Setdb2 may not only regulate antibacterial responses but also modulate genes encoding molecules involved in disease tolerance in superinfection.

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**Online Methods**

**Mice.** C57BL/6J mice (wild-type) were obtained from The Jackson Laboratory, and Ifnar1<−/− mice, IfnγR−/− mice, IfnγR−/− Stat1−/− mice and Th2−/−Cd56−/− mice were on a C57BL/6J background. To generate Setdb2<ΔGT/GT> mice, the targeting vector pEFK106 was made in *Escherichia coli* strain EL350 by recombination-mediated genetic engineering with the Lambda RED system.5,6 A genomic phage artificial chromosome library (i.e., from the 129Sve/TACBe genetic background; Roswell Park Cancer Institute mouse phage artificial chromosome library 21; Medical Research Council Geneservice) was screened for the full-length Setdb2 gene with specific cDNA probes. A positive clone, RP21-498K23, was used for recovery of flanking genomic sequences. We generated a 2.3-kilobase genomic fragment introducing an EcoRV restriction site andloxP sites and a cassette for resistance to neomycin (neo) with plasmid pLMJ237, for insertion downstream of exon 3. neo was removed by induction of Cre recombinase with arabinoside. Similarly, a 2.9-kilobase genomic fragment harboring the gene-trap cassette was generated with a Fp- recognition target andloxP sites, as well as a heterologous BarnHI restriction site, was generated by recombination-mediated genetic engineering with pLM330. The gene-trap cassette contains a pGK/EM7 dual promoter, a strong translation site, was generated by recombination-mediated genetic engineering with pLM330. The gene-trap cassette is a PVR-digested DNA (Supplementary Fig. 3b). Positive embryonic stem cell clones were microinjected into blastulae and were then transferred to pseudopregnant mice by standard methods. Mice were genotyped for the presence of the gene-trap cassette by Southern blot hybridization (data not shown) and by amplification of genomic DNA by PCR with the primers 5′-AATGCGGCAATTTGATAGAAGC-3′ and 5′-GATCTTGCTAAAGGTCACCA-3′ (Supplementary Fig. 3c). The wild-type allele ampiclon was a PCR product of 422 base pairs, while the knocked-in gene-trap ampiclon was a PCR product of 516 base pairs. All Setdb2<ΔGT/GT> mice used in this study were backcrossed for over ten generations onto a C57BL/6J background.

All mice were kept under specific pathogen-free conditions at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences, the Medical University of Vienna, National Cancer institute, the Ohio State University, and/or the Institute for Systems Biology, Seattle. For all experiments, sex- and age-matched mice were used. The animal protocols were approved by the Interventional Animal Care and Use Committees of the National Cancer Institute-Frederick, the Ohio State University and the Institute of Systems Biology in Seattle and by the ethical committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research.

**BMDMs.** BMDMs were isolated from C57BL/6J, Ifnar1<−/−> and Setdb2<ΔGT/GT> mice and were cultured in RPMI medium containing 10% FBS, penicillin-streptomycin-glutamine (10378-016; Life Technologies) and 50 ng/ml recombinant mouse macrophage colony stimulating factor (34-8983-85; eBioscience). BMDMs were stimulated with PAM3CSK4 (tripalmitoyl cystitial seryl tetraslyne lipopetide; 500 ng/ml;tlrl-pms; Invivogen), poly(I:C) (6 µg/ml; 4635-ML-025; Biomedica). For the experiments in Supplementary Figures 2b and Supplementary Table 1, RNA was processed for hybridization to the mouse genome assembly GRCm38 of the Genome Reference Consortium (assembly mm10 of the UCSC Genome Browser) with the TopHat splice-junction mapper (version 2.0.12) using the mouse gene and transcript annotation from the Ensembl project of genome databases version 70 as reference transcriptome. The TopHat ‘max-multihits’ option was set to 100, while the length (L) of the seed substrings of the underlying Bowtie 2 aligner software (version 2.2.3) were reduced from 20 to 14. Programs from the Cufflinks software package (version 2.2.1) were used to assemble transcripts, merge transcript assemblies of replicates and samples before final testing for differences in expression with the Cuffdiff program. The default false-discovery rate of 0.05 was left unchanged. Cuffdiff comparisons were post-processed, and quality assessment plots were drawn with the Bioconductor package curremrbund (version 2.6.1).

**Bioinformatics analyses.** Gene-ontology enrichment analyses were done with DAVID Bioinformatics Resources 6.7 and the Molecular Signatures Database of Gene Set Enrichment Analysis. Gene Set Enrichment Analysis of transcription factor–binding targets was performed through the use of transcription factor–binding sites as defined in the TRANSFAC public database, version 7.4. The computed robust multi-array average values were subjected to significance analysis of microarray with Microarray expression Viewer v4.9. A two-class paired test with default parameters was used and calculated the Δ value and a cutoff of 3 to identify genes with significant regulation. Heat maps were plotted with software of the R project for statistical computing. Motif scanning for transcription factor–binding sites was performed 3 kilobases upstream and downstream of the transcriptional start site of Setdb2 as described.

A list of NF-kB target genes was compiled with resources from the websites of The Gilmore Lab, Boston University (http://www.bu.edu/nf-kb/ gene-resources/target-genes/) and the Institut de Biologie de Lille et LIFL (http://bioinfo.lifl.fr/NF-KB/#haut%20de%20page), as well as from published literature. The resulting total list of 373 NF-kB target genes (Supplementary Table 1) was used to calculate the hypergeometric distribution (with the assumption of a total number of 24,561 coding genes; source, Mouse Genome Informatics) of overlapping genes in the protein-coding genes with significant upregulation of >1.5-fold at one time point or more (P < 0.001).

**Real-time PCR.** For the measurement of gene expression by real-time PCR, total RNA was isolated with QIAzol lysis reagent (Qiagen) and was reverse-transcribed with a First Strand CDNA Synthesis Kit (Fermentas). Subsequently, gene expression was analyzed with Taqman Fast Universal PCR Mastermix and Taqman Gene Expression assays (Setdb2, Mm01318748_m1, Cxcl1, Mm00435389_m1; and I6, Mm00946190_ml) (Life Technologies), as well as an assay for the gene encoding M protein of influenza virus A/PR/8/34 with oligonucleotides 5′-CATGGAATTGCTAAGGACAGGAC-3′ (forward) and 5′-CCATTAAAAGGACATTGGACA-3′ (reverse) and the Taqman probe 5′-FAM-TTGTGTCCACCGCCATGGCGTTCA-BHQ1-3′. Real-time PCR experiments were run on a 7900HT Real-Time PCR system or a StepOnePlus Real-Time PCR system (Life Technologies). Expression data were normalized to those of the housekeeping gene *Eef1a1* (encoding eukaryotic translation elongation factor 1 α).
Generation of a Setdb2-specific monoclonal antibody. A carboxy-terminal 60–amino acid sequence (amino acid positions 541–600) was fused into a hepatitis B virus carrier protein as an immunogen. This region was amplified by PCR and inserted into a six-histidine-tagged plasmid. The fusion protein was expressed in E. coli BL21 and was purified on 1-mL HisTrap HP columns (GE Healthcare), followed by a linear imidazole gradient on an ÄKTA FPLC system (GE Healthcare). The fractions were separated by SDS-PAGE and were concentrated with Amicon Ultra 15-3K Centrifugal Filter Devices (Millipore). The immunization and generation of monoclonal B-cell hybridomas was performed by challenging of Setdb2 mice subcutaneously three times (every 2 weeks) with 50 μg of purified fusion protein antigen mixed at a ratio of 1:1 with adjuvant, before a final intravenous immunization with 50 μg purified antigen (adjuvant free). Mouse serum and clone pools were tested by immunoblot analysis for the detection of overexpressed and endogenous mouse Setdb2. Clone 7H7F11 yielded the best signal-to-noise performance.

Immunoblot analysis. Protein concentrations of cell lysates and organs were determined with a Coomassie Protein Assay kit (Thermo Scientific). Proteins were separated by SDS-PAGE with NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies) and were transferred to nitrocellulose membranes. Proteins were detected with Pierce ECL Western Blotting Substrate (Thermo Scientific) or Amersham ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences). Gels were visualized with the chemiluminescent gel documentation system F-C hemiBlot (DNR Bio-Imaging Systems).

ELISA. Protein concentrations were determined with a Mouse CXCL1/KC Quantikine ELISA Kit (MKC00B) or Mouse CXCL1/KC DuoSet (DY453), a Mouse CXCL2/2 DuoSet (DY452), a Mouse IL-6 DuoSet (DY406) (all from R&D Systems) or a Mouse BD OptEIA IL-10 kit (555252; BD Biosciences). ELISAs were performed according to the manufacturer's instructions.

ChIP analysis. BMDMs derived from wild-type or Setdb2 mice were left untreated or treated for 2 h with poly(I:C). The same number of cells in each experimental condition were subsequently harvested, fixed for 10 min at room temperature with 1% formaldehyde and lysed in 1% SDS buffer. Chromatin was sheared to an average size of 300 base pairs with an S2X Focused-sonicator (Covaris), and the amount of chromatin was adjusted by measurement with an ND-1000 spectrophotometer (NanoDrop) to match wild-type and Setdb2 samples before ChIP analysis of histone marks. The chromatin prepared from wild-type or wild-type and Setdb2 samples was subjected to ChIP with anti-setdb2 (7H7F11; described above), anti-H3K9me3 (Ab8898; Abcam) or anti-H3K9ac (07-352; Millipore) according to a published procedure50 modified by the use of magnetic Dynabeads Protein-G beads (Life Technologies). For mock ChIP controls, empty beads were used. For mock controls in ChIP analysis of Setdb2, the method used for culture of the hybridoma 7H7F11 was added instead. The ChIP efficiency was controlled by quantitative real-time PCR analysis with the following primers: Cxcl1 promoter region, 5′-CTCTCCTCATGCTCCTCCGG-3′ (forward) and 5′-CGGGGATGAATCCTTCTTTG-3′ (reverse); Cxcl1 exon 1 region, 5′-GGTCCGACGCTACCCAGTCTCC-3′ (forward) and 5′-AGTGCCGAGACCTGCCGTCC-3′ (reverse); Atpb promoter region, 5′-CCCTGCTGGTTGGATGTGCAC-3′ (forward) and 5′-CTTCACCTATACCCGGGCGC-3′ (reverse).

LPS-induced pulmonary neutrophilia model. Mice were anesthetized with ketamine and xylazine and were given intranasal administration of 0.4 μl of LPS (E. coli serotype 0111:B4, L391; Sigma). Four hours later, mice were killed and BAL fluid was obtained by washing of the lungs three times with PBS in a total volume of 1 ml. Total cells in BAL fluid were counted with an improved Neubauer hemocytometer. Cyto centrifuged preparations (Cytospin-4; Shandon Instruments) were stained with Diff-Quik (Thermo Fisher Scientific), and the percentage of inflammatory cells was determined by morphological examination of at least 300 cells per sample.

Infection models. For the lung analyses in Figure 1, mice were anesthetized with ketamine and xylazine and were intranasally infected with 15 μl or 50 μl of PBS containing ~1 × 10⁴ plaque-forming units of influenza virus A/PR/8/34 (PR8) (originally obtained from Charles River Laboratories). In all other experiments, mice were infected intranasally with a sublethal dose of influenza virus PBS (~1 × 10⁴ plaque-forming units) or the appropriate dose of S. pneumoniae strain ATCC 6303. Samples for the 16-hour time point in Figure 5 were harvested in the time span corresponding to 14–16 h after superinfection. Bacterial titers were determined from lung homogenates by plating of tenfold serial dilutions on blood agar plates. Lung wet weights were determined with a Pioneer precision balance (Ohaus).

Flow cytometry. Lung tissue was harvested and single-cell suspensions were prepared with a metal mesh. For calculation of absolute cell numbers, cells were counted with an improved Neubauer hemocytometer. Single-cell suspensions of the lungs and collected BAL fluid cells were incubated with CD16/CD32 Fc Block (93; 101310; BioLegend) to prevent nonspecific antibody binding. For flow cytometry, cells were stained with the following antibodies: anti-B220/CD45R (RA3-6B2; 45-0452; eBioscience), anti-CD3e (145-2C11; 100320; BioLegend), anti-CD11b (M1/70; 101206; BioLegend), anti-Ly-6G (18-7-9686; eBioscience), anti-CD45 (30-F11; 103137; BioLegend), anti-CD11c (N418; 117333; BioLegend) and anti-SiglecF (E50-2440; 562681; BD). For exclusion of dead cells from the analysis, samples were labeled with the Fixable Viability Dye eFluor 780 (65-0655; eBioscience).

Histology. Lung tissue was fixed with either 4% paraformaldehyde or 10% formalin and were embedded in paraffin. Immunohistochemistry was performed on sections 3–4 μm in thickness. Endogenous peroxidase was neutralized (3% H₂O₂ in PBS), and nonspecific binding was blocked (10% FCS in PBS). Sections were then incubated overnight at 4 °C with goat antibody to influenza virus (5315-0064; Serotec). Bound primary antibody was visualized by a biotin technique with 3,3′ diaminobenzidine as the chromogen (with counterstaining of nuclei with hemalaun).

In Figure 5h, histology scores were assigned by a trained pathologist, blinded to group identity, for lung sections stained with hematoxylin and eosin. The severity of inflammation and pneumonia was evaluated on the basis of the presence of interstitial inflammation, alveolar inflammation, pleuritis, bronchitis and endothelitis by the following scoring system: 0, absence; 1, mild; 2, moderate; and 3, severe. Additionally, a score of 1 was added for the presence of pneumonia, edema or thrombi formation, and a score of 0.5 was added for every infiltrate covering 10% of the lung area. The sum of all parameters indicates the total histology score.

Statistical analysis. Statistical differences between experimental groups were determined by an unpaired t-test. If variances (F-test) between the compared data sets were significantly different, an unpaired t-test with Welch’s correction was performed. P values of ≤0.05 were considered significant. Graphs and statistical tests were made with GraphPad Prism version 6.

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