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Graphical abstract

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Glucocorticoid receptor dimers control intestinal STAT1 and TNF-induced inflammation in mice

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Introduction
The cytokine TNF is of great interest in biomedical research. Low but chronic expression levels of TNF have been shown to be sufficient for the development of diseases that have a large economic and social impact, e.g., rheumatoid arthritis and inflammatory bowel diseases (IBDs), such as Crohn disease. For the treatment of these and other diseases, TNF-inhibiting biological drugs have been shown to be a good option for patients not responding to first-line treatments, for example, with methotrexate (1). Although anti-TNF treatment is often successful, there are a number of problems associated with anti-TNF therapy, such as side effects, nonresponsiveness, and high cost (1). Interestingly, in such chronic diseases, acute exacerbations leading to inflammation flares are observed that may be caused by several molecules, including damage-associated molecular patterns (DAMPs) and cytokines and even an acute increase in TNF production (2). Therefore, understanding the impact, mechanism, and therapeutic inhibition of acute TNF-induced toxicity is essential.

Although there is debate about their underlying mechanism of action, glucocorticoids (GCs) are used in the management of several acute inflammatory conditions. TNF-induced acute lethal inflammation represents an interesting mouse model, in which intestinal epithelial cells (IECs) have been described as the main target cells of TNF (3–5). In mice, at least 2 gut-associated mechanisms contribute to loss of IEC barrier function, which is a crucial step in TNF-induced lethality: (a) loss of mucus in goblet cells and loss of antibacterial granules in Paneth cells and (b) damage and cell death in the IEC layer (5, 6).

GCs protect against TNF-induced lethal shock and TNF-induced intestinal permeability (5). GCs, such as dexamethasone (DEX), bind and activate the glucocorticoid receptor (GR) (7). They are used to treat a variety of inflammatory disorders, such as rheumatoid arthritis and IBDs (8). After binding, GR moves to the nucleus and regulates gene transcription as a monomer or as a homodimer. For a long time, the antiinflammatory effects of GCs were believed to result mainly from tethering (i.e., protein-protein interactions) of monomeric GR to inflammatory transcription factors, such as NF-κB and AP-1. However, more recent research indicates that GR homodimers, which form a DNA-binding transcription factor, are essential for mediating the antiinflammatory properties of GCs in acute inflammatory settings, such as systemic inflammatory response syndrome (SIRS) (9, 10). Most of these data are based on findings in GRhom/hom mice. These mice express a mutant version of GR, carrying a missense point mutation (A458T) that leads to reduced GR dimerization and DNA binding, yet maintains an intact monomer profile (11, 12). Upon injection of synthetic GCs, GRhom/hom mice show significantly reduced expression of GC response element (GRE) responsive

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target genes (9, 13, 14). The GRdim/dim mutant protein binds much
and DEX-stimulated IECs of GR WT/WT and GR dim/dim mice. In non-
measured genome-wide mRNA expression profiles of PBS-, TNF-
TNF-mediated pathological changes at the level of the IECs. We
the Dusp1 gene encoding the IL1 receptor antagonist in the endo-
ance of GR WT/WT villi, but villi damage, shortening, cell death, and loss of
goblet cells in GR dim/dim as well as TUNEL signals at villi tops and crypts.

Results

Intestinal epithelial GR and GR dimers play an essential role in protection
against TNF-induced lethality, gut permeability, and cell death. We
previously showed that GRdim/dim mice display a significant increase in sensitivity toward TNF-induced lethality and that TNF lethality is linked with apparent loss of goblet cells and Paneth
cells and permeability of the IECs (5, 9). Hence, we decided to
study the role of GR dimers in the intestinal epithelium during
TNF-induced SIRS. GRVilinKO mice, which selectively lack a func-
tional GR-coding gene (Nr3c1) in IECs, were used (16). GRVilinKO
and control GR+/+ mice were injected with a single dose of 35 μg
TNF, and survival was monitored. GRVilinKO mice were significantly
more sensitive compared with the control GR+/+ mice (Figure 1A).
It has been known for a long time that injection of GCs protects
against TNF-induced lethal shock (7). Interestingly, DEX could
not protect GRVilinKO mice (Figure 1A), strongly indicating that
protection by DEX requires GR expression in IECs.

To investigate the importance of GR dimers in protection against
TNF lethality by exogenous GCs, we pretreated GR WT/WT
and GRdim/dim mice with 10 mg/kg DEX or PBS, followed by increasing
doses of TNF. Survival was monitored (Figure 1B), and an LD50
was defined for all mouse groups (Figure 1C). GRdim/dim mice
displayed a higher sensitivity to TNF compared with GR WT/WT
mice, with LD50 values of, respectively, 10 μg and 30.5 μg per mouse. In
GR WT/WT mice, a single injection of DEX increased the LD50 of
TNF over 3 times (from 30.5 μg to 96.8 μg per mouse), but in GRdim/dim
mice, DEX had no significant protective effect and increased the
LD50 from 10 μg to 11.9 μg per mouse. Our data suggest an essential
role for IEC GR and, in particular, an optimal dimerization func-
tion in resistance against TNF-induced lethal shock, in both the
absence and presence of exogenous GCs.

Next, we investigated whether GR dimers in IECs protect
against TNF by reducing intestinal permeability previously shown
to be strongly linked with TNF lethality (5). We studied the intesti-
nal permeability and cell death of IECs in the ileum of GR WT/WT
and GRdim/dim mice after TNF challenge. In both groups of mice, we
used 12.5 μg TNF, which is a lethal dose in GRdim/dim mice, but is not
lethal in GR WT/WT mice. In contrast to GR WT/WT mice, GRdim/dim
mice displayed significant increased intestinal permeability determined via FITC-dextran leakage into the blood upon oral gavage (Figure 1D) and bowel damage as judged by H&E staining after TNF challenge
(Figure 1E). GRdim/dim mice also displayed TNF-induced cell death
of IECs, while this was virtually absent in GR WT/WT mice, as
quantified by TUNEL stainings. Positive red signals were particu-
larly prominent at the villi tops and less so in the crypts (Figure 1F).

GR dimers are essential for suppressing STAT1 expression and
activity specifically in the gut. In order to investigate why GRdim/dim
mice are sensitive to these TNF-induced effects on IECs, we
performed an RNA-sequencing (RNA-seq) analysis of IECs of
GR WT/WT and GRdim/dim mice 8 hours after injection with PBS or
with TNF. In control PBS-injected mice, 460 and 240 genes
were identified, suggesting that necroptosis is a process promoted by the microbiome
and IFNs and controlled by dimeric GR. Therefore, we conclude
that under physiological conditions, the microbiota sustain a local
GC production in order to prevent a detrimental ISG expression
signature induced by the very same microbiota in a GR dimer-
dependent way. Failure of this protective loop leads to extreme
sensitization for cell death in IECs and TNF-induced gut perme-
ability and subsequent lethality.
enrichment of ISRE, IRF1, and IRF2 motif–containing genes in IECs of naïve GRdim/dim mice. (A–C) RNA-seq of IECs of GRWT/WT (black) and GRdim/dim (white) mice (n = 3 per group). (A) HOMER motif analysis of DE genes in GRdim/dim compared with GRWT/WT mice. Motifs with highest rank and their P values and q values are displayed. (B) Heat map of DE genes containing an ISRE and/or IRF-1 motif. (C) Confirmation of RNA-seq data with qPCR on independent new samples (n = 3 per group). Ifit1, Ifi84, Ifi15, and Stat1 mRNA expression are shown as mean ± SEM. P values were calculated using Student’s t test. (D–F) GRWT/WT and GRdim/dim mice received drinking water with or without antibiotics for 3 weeks, after which IEC samples were taken (n = 3 per group). (D) STAT1 and p-STAT1 protein levels were analyzed by Western blot using actin as a loading control. (E) Relative STAT1 and p-STAT1 signal intensities were quantified and normalized to ACTIN and STAT1 levels respectively. P values were calculated using 2-way ANOVA. (F) STAT1 and Ifit1 mRNA expression were determined in IECs of GRWT/WT (black) and GRdim/dim (white) mice using qPCR and are shown as mean ± SEM (n = 5 per group). P values were calculated using Student’s t test. (G) GR recruitment to 2 IR-nGRE sites in the STAT1 promoter (IR-nGRE1 and IR-nGRE2). GRWT/WT (black) and GRdim/dim (white) mice were treated with PBS or 10 mg/kg DEX for 2 hours (n = 5 per group; combined data of 3 independent experiments). ChIP on IEC samples was performed against GR using an H300 antibody. Data were normalized to input for each sample and expressed as fold change of H300 to IgG control. P values were calculated using 2-way ANOVA. ***P < 0.0001; **P < 0.001; *P < 0.05; *P ≤ 0.05.

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Figure 2. ISGs are expressed in the IECs of naïve GRdim/dim mice. (A–C) RNA-seq of IECs of GRWT/WT (black) and GRdim/dim (white) mice (n = 3 per group). (A) HOMER motif analysis of DE genes in GRdim/dim compared with GRWT/WT mice. Motifs with highest rank and their P values and q values are displayed. (B) Heat map of DE genes containing an ISRE and/or IRF-1 motif. (C) Confirmation of RNA-seq data with qPCR on independent new samples (n = 3 per group). Ifit1, Ifi84, Ifi15, and Stat1 mRNA expression are shown as mean ± SEM. P values were calculated using Student’s t test. (D–F) GRWT/WT and GRdim/dim mice received drinking water with or without antibiotics for 3 weeks, after which IEC samples were taken (n = 3 per group). (D) STAT1 and p-STAT1 protein levels were analyzed by Western blot using actin as a loading control. (E) Relative STAT1 and p-STAT1 signal intensities were quantified and normalized to ACTIN and STAT1 levels respectively. P values were calculated using 2-way ANOVA. (F) STAT1 and Ifit1 mRNA expression were determined in IECs of GRWT/WT (black) and GRdim/dim (white) mice using qPCR and are shown as mean ± SEM (n = 5 per group). P values were calculated using Student’s t test. (G) GR recruitment to 2 IR-nGRE sites in the STAT1 promoter (IR-nGRE1 and IR-nGRE2). GRWT/WT (black) and GRdim/dim (white) mice were treated with PBS or 10 mg/kg DEX for 2 hours (n = 5 per group; combined data of 3 independent experiments). ChIP on IEC samples was performed against GR using an H300 antibody. Data were normalized to input for each sample and expressed as fold change of H300 to IgG control. P values were calculated using 2-way ANOVA. ***P < 0.0001; **P < 0.001; *P < 0.05; *P ≤ 0.05.

The induction of ISGs, such as Ifit1, is under the control of the transcription factor complex IFN-stimulated gene factor 3 (ISGF3), which consists of STAT1, STAT2, and IRF9 (18). In agreement with the fact that many of the STAT1-responsive ISG genes are upregulated in GRdim/dim mice, suggesting an active ISGF3 complex, we found that STAT1 protein levels were increased in GRdim/dim mice compared with GRWT/WT mice and that STAT1 was only phosphorylated in the IECs of GRdim/dim mice (Figure 2, D and E). Furthermore, an ISG signature was detected in the IECs of GVRillinKO mice (Figure 2F), suggesting that the ISG signature is mediated by a lack of local (dimeric) GR signaling.

To confirm that the ISG signature in IECs of GRdim/dim mice is also observed at the protein level, we performed a proteome-wide mass spectrometry (liquid chromatography–tandem mass spectrometry [LC-MS/MS]) experiment on ileum samples of GRWT/WT and GRdim/dim mice. Differential protein concentrations with a log2 fold change of 1.0 and an adjusted P value of 0.05 are listed in Supplemental Figure 2. We found 32 proteins upregulated in GRdim/dim, including 17 known as IFN stimulated (18) (17/32 significant enrichment of IFN proteins, P = 0.003), including STAT1.

A conceivable interpretation of our data is that GR mediates repression of ISGs via the control of STAT1 expression in a GR dimer–dependent way. To repress gene expression in a GR dimer–dependent way, the gene should contain one or more negative or inhibitory GREs in its promoter. By motif analysis, we identified 2 so-called IR-nGRE elements, as previously defined (15), in the promoter of STAT1: IR-nGRE1 with sequence CACCTGGAGA and IR-nGRE2 with sequence CTCCAGGACA at positions –2841 and –757 relative to the transcription start site, respectively. We studied GR binding to these elements via ChIP on ileum tissue extracts (Figure 2G). In the graph, data are represented as the fold change of the GR-specific H300 antibody to the IgG control, thus showing specific recruitment of GR to both IR-nGRE elements in the STAT1 promoter (ratio GR to IgG control). In basal conditions, GR was recruited to the IR-nGRE elements in the STAT1 promoter in both GRWT/WT mice and GRdim/dim mice, as shown by a 2-fold change. Interestingly, GR was substantial more recruited to these elements upon DEX treatment in GRWT/WT mice compared with DEX-treated GRdim/dim mice. These data potentially validate the IR-nGRE elements in the STAT1 promoter as GR dimer–bound elements that might be involved in the GR dimer–dependent repression of Stat1 gene expression.

The IEC-specific ISG signature and corticosterone production in GRdim/dim mice are modulated by the gut microbiome. Interestingly, the increased expression of Stat1 mRNA observed in GRdim/dim mice was only observed in the intestinal epithelium and not in other organs, such as liver, macrophages, spleen, and adrenal glands (Figure 3A). The gut is a unique organ in which local GCs are produced and the gut microbiota are present (19). Microbiota are known to trigger signaling pathways, leading to the production of IFNs and activation of their signaling cascades (20–22). We hypothesize that, under basal GR WT conditions, IFN signaling in IECs is stimulated by the microbiota, but that this IFN response is controlled by local GC production that leads to low but important GR dimer–repressor activity.

To investigate this hypothesis, we studied the effect of the microbiome on basal gene expression in the IECs of GRWT/WT and GRdim/dim mice. Both groups of mice were treated with an antibiotic cocktail (+AB) for 3 weeks to deplete the bacteria, which was confirmed by plating out stool samples. In both groups of mice, antibiotic treatment reduced Stat1 and Ifit1 expression in IECs (Figure 3, B and C). Following mRNA expression, total STAT1 protein levels were reduced in both groups of mice after antibiotic treatment. In addition, a downward trend in the levels of phosphorylated STAT1 (p-STAT1) was observed in GRdim/dim mice only (P = 0.07), as there are no p-STAT1 levels measurable in GRWT/WT mice (Figure 2, D and E). Interestingly, in GRdim/dim mice, the levels of Stat1 and Ifit1 mRNA after antibiotic treatment remained higher than in untreated GRWT/WT mice, suggesting not only that the triggering of TLRs or IFN-β production by the antibiotic-sensitive microbiota induces the ISG signature in GRdim/dim mice, but also that additional factors, e.g., antibiotic-resistant bacteria, viruses, and fungi, are involved. The reduction in the ISG signature by antibiotics was somewhat protected in the ISG signature in GRWT/WT and GRdim/dim mice were found to be partly protected (Figure 3D).

Next to adrenal cortical cells, IECs are known to produce GCs (19). Therefore, local stimulation of corticosterone (CS) production by microbiota components could be an additional factor needed to control the ISG signature in the IECs. To address this,
we first studied the impact of antibiotics on the expression of the Cyp11a1 and Cyp11b1 genes, which encode critical steroidogenic enzymes involved in CS synthesis, in IECs of GRWT/WT and GRdim/dim mice. In both mouse groups, the expression of these genes was reduced in IECs of mice subjected to antibiotics, supporting our hypothesis (Figure 3, E and F). Interestingly, the expression of Cyp11a1 and Cyp11b1 genes was higher in GRdim/dim mice compared with GRWT/WT mice. This correlates with the fact that both genes are known ISG genes and are found in the Interferome database (17). Next, the local production of CS in IECs in the absence of microbiota was determined. Interestingly, ex vivo CS production by ileum explants was significantly reduced by antibiotic treatment in mice (Figure 3G). Serum CS levels, which are higher in GRdim/dim than GRWT/WT mice (23), were also reduced by about half by antibiotics (data not shown). Together, these data support our hypothesis that microbiota stimulate local CS production and activate GR dimers, which are essential in controlling the microbiota-stimulated ISG signature in the gut.

**STAT1 and its responsive genes are master regulators of sensitivity to TNF.** STAT1-deficient (STAT1−/−) mice exhibit strong IFN-signaling defects and do not display any of the physiological responses associated with IFNs (24–26). In contrast to STAT1+/− mice, full STAT1−/−
mice fail to express *Ifit1*, *Ifit2*, or other ISGs (including *Cyp11a1* and *Cyp11b1*) in IECs in unstimulated conditions (Figure 4A). STAT1−/− mice were found to resist a lethal dose of TNF (Figure 4B) as well as TNF-induced intestinal permeability and cell death (Figure 4, C and D). These data support the idea that STAT1 is a crucial regulator of TNF-induced intestinal permeability, IEC cell death, and subsequent lethality. Unfortunately, homozygous STAT1−/− GRdim/dim double-mutant mice were not viable. To investigate whether the upregulated and phosphorylated intestinal STAT1 is a master regulator of GRdim/dim hypersensitivity to TNF, we administered the JAK/STAT inhibitor tofacitinib (27). Tofacitinib (100 mg/kg) or vehicle was given orally to GRdim/dim mice (n = 10 per group; combined data of 2 independent experiments). Mice received tofacitinib twice a day for 2 days before challenge (20 μg TNF) and 2 times (1 hour before and 8 hours after) on the day of challenge. Survival was monitored and analyzed with a log-rank test. (E) LFCs of TNF-induced ISGs, detected by RNA-seq, in IECs of GRWT/WT mice (black) or GRdim/dim mice (white) injected with 12.5 μg TNF (n = 4 per group). (F) Ifit1 and Ifit2 mRNA expression, measured by qPCR, in IECs of GRWT/WT and GRdim/dim, 8 hours after PBS or 12.5 μg TNF (n = 4 per group). P values were calculated using 2-way ANOVA. All bars represent mean ± SEM. ****P < 0.0001; ***P < 0.001; **P ≤ 0.01; *P ≤ 0.05.
In both GRWT /WT and GRdim/dim mice, a dose of 12.5 μg TNF induced an almost identical set of ISGs, but TNF induction of these genes was significantly higher in GR dim/dim than in GRWT /WT mice (Figure 4F, log y axis). This is also illustrated by the expression pattern of Ifit1 and Ifit2 (Figure 4G). Together, these results suggest a poorly controllable ISG induction in GRdim/dim mice upon TNF challenge. Based on the data with STAT1-deficient mice (Figure 4C) and our previous work using IFN- and IFN-receptor-1–deficient mice (3), the insufficient control of ISG induction in GRdim/dim mice is likely to form the basis of their hypersensitivity to TNF-induced lethality.

Figure 5. Lack of protective effects of DEX against TNF-induced changes in the gut of GRdim/dim mice. (A–C) GRWT/WT (black) and GRdim/dim (white) mice were pretreated with vehicle or 10 mg/kg DEX for 30 minutes, followed by challenge with 50 μg or 12.5 μg TNF, respectively. (A) Eight hours after TNF challenge, systemic concentrations of FITC-dextran in plasma were determined after oral gavage and reflect relative intestinal permeability (n = 12–20 per group; combined data of 3 independent experiments). (B) Ileum was sampled 2 hours and 8 hours after TNF injection (n = 5 per group), then stained with H&E and bowel damage scores determined. (C) Sections were stained for TUNEL and quantified. All bars represent mean ± SEM. P values were calculated using 2-way ANOVA. (D–F) RNA-seq results of IECs of GRWT/WT and GRdim/dim mice pretreated with 10 mg/kg DEX or PBS, followed 30 minutes later by a lethal dose of TNF (50 μg for GRWT/WT and 12.5 μg for GRdim/dim mice) or PBS. (D) Overview of the experimental protocol. For all groups, n = 3 per group. (E) Venn diagram of DE genes upon DEX treatment compared with PBS-stimulated conditions. (F) Overview of the most significant transcription factor–binding motifs found enriched in the DEX-induced (left panel) and DEX-reduced (right panel) genes in GRWT/WT mice, using HOMER. Motifs with the highest rank and their P values and q values. ****P < 0.0001; ***P < 0.001; **P ≤ 0.01; *P ≤ 0.05.

gene-expression profiles in IECs of GRWT/WT and GRdim/dim mice challenged with different doses of TNF. Mice were injected with 12.5 μg TNF, which is a sublethal dose in GRWT/WT mice (referred to as WT12.5), but is a lethal dose in GRdim/dim mice (DIM12.5). GRWT/WT mice were also challenged with a lethal dose (50 μg) of TNF (WT50). A comparison between the response to different doses of TNF is discussed in Supplemental Figure 3. Ingenuity Pathway Analysis (IPA) revealed that the major pathway activated by lethal doses of TNF in GRWT/WT and GRdim/dim mice is the IFN-signaling pathway. When focusing on all 2,029 genes that are significantly induced by TNF (across the 3 groups), 736 of these genes were identified as ISGs by the Interferome database (17). In both GRWT/WT and GRdim/dim mice, a dose of 12.5 μg TNF induced an almost identical set of ISGs, but TNF induction of these genes was significantly higher in GRdim/dim than in GRWT/WT mice (Figure 4F, log y axis). This is also illustrated by the expression pattern of Ifit1 and Ifit2 (Figure 4G). Together, these results suggest a poorly controllable ISG induction in GRdim/dim mice upon TNF challenge. Based on the data with STAT1-deficient mice (Figure 4C) and our previous work using IFN- and IFN-receptor-1–deficient mice (3), the insufficient control of ISG induction in GRdim/dim mice is likely to form the basis of their hypersensitivity to TNF-induced lethality.
Lack of protective effects of exogenous GCs against TNF-induced changes in the gut of GRdim/dim mice. To study the effect of exogenous GCs on TNF-induced intestinal permeability, we pretreated mice with vehicle or DEX (minus 30 minutes) and challenged GRWT/WT and GRdim/dim mice with a lethal dose of TNF, 50 μg and 12.5 μg, respectively. Eight hours after TNF challenge, the TNF-induced intestinal permeability was significantly decreased by DEX in GRWT/WT mice, but not in GRdim/dim mice (Figure 5A). To determine the damage of the intestine, ileum was sectioned 2 hours and 8 hours after TNF challenge and damage was quantified visually using the validated protocol (28), including scores for villus length, villus erosion, cell death, and loss of goblet cells (Figure 5B). TNF caused massive damage to the gut in all samples. DEX was able to protect partially against the TNF-induced damage to the villi in GRWT/WT mice, but not in GRdim/dim mice. TUNEL staining on ileum sections 8 hours after TNF challenge revealed more TUNEL activity induced by TNF in GRdim/dim compared with GRWT/WT mice. Moreover, DEX had a significant protective effect in GRWT/WT mice, but not in GRdim/dim mice (Figure 5C).

GR dimer–specific repression of ISGs, including necroptosis master switches Ripk3, Zbp1, and Mlkl. To identify the biological pathway behind the observed GR dimer–dependent protective effects in Figure 5A–C, we studied the effects of DEX or PBS pretreatment (minus 30 minutes) on gene expression by RNA-seq of GRWT/WT and GRdim/dim IECs 8 hours after TNF or PBS. An overview of the numbers of DE genes (up and down) under DEX conditions, relative to noninduced (PBS injected) levels is provided in Figure 5E. No specifically enriched motifs were found by HOMER in the GRdim/dim–specific regulated genes. As expected, one of the prominent enriched motifs found in the family of GRWT/WT–specific DEX-induced genes is the classical GRE. Interestingly, the major motifs found in GRWT/WT–specific DEX downregulated genes are ISRE and IRF motifs (Figure 5F).

A dimer-specific repression of ISGs seems a plausible mechanism underlying the molecular basis of the GR dimer–specific protection of DEX against TNF-induced gut permeability and lethality. To investigate whether DEX represses TNF-induced ISRE genes more prominently in GRWT/WT compared with GRdim/dim mice, we studied Stat1 and Ift1 gene-expression profiles in both mouse groups (Figure 6, A and B). Although DEX is able to repress TNF-induced expression of these genes, the process is less efficient in GRdim/dim mice compared with GRWT/WT mice. As mentioned before, we found DEX-stimulated GR recruitment to both IRα-GR elements in the STAT1 promoter upon DEX treatment, but only in GRWT/WT mice (Figure 2G). When evaluating the impact of DEX on transcriptional levels of TNF-induced ISGs specifically in GRWT/WT and GRdim/dim mice, we focused on 736 ISGs (induced by TNF and identified by the Interferome database; ref. 17) and studied the impact of DEX on the log-fold induction of these genes. As shown in Figure 6C, the reduced expression by DEX was found to be stronger in GRWT/WT mice compared with GRdim/dim mice (35% and 28%, respectively), suggesting poorer repression of ISGs by DEX in GRdim/dim mice. Next, we questioned whether DEX was able to significantly repress TNF-induced ISGs in GRWT/WT mice, but not in GRdim/dim mice, and found a list of 93 genes following this pattern (Figure 6D and Supplemental Table 3). Interestingly, this list contains genes coding for proteins important in the induction of necroptotic cell death, namely Ripk3, Zbp1, and Mlkl.

The expression levels of these 3 genes, as determined by RNA-seq, are depicted in Figure 6E (Ripk3) and Supplemental Table 3 (Zbp1 and Mlkl). Based on the data in Figure 6E, it is clear that Ripk3 is significantly more highly expressed in GRdim/dim mice compared with GRWT/WT mice in basal conditions and after stimulation with an equal dose of TNF (12.5 μg/mouse) in both groups. Moreover, expression of Ripk3 mRNA is less repressed by DEX pretreatment in these mice. The function of these genes is strictly linked to necroptotic cell death (29). The induction of RIPK3 protein was measured by IHC on ileum sections (Figure 6F) and appeared more pronounced in the gut of GRdim/dim compared with GRWT/WT mice. The staining was particularly strong in crypts. Supporting a role for necroptosis, the extreme sensitivity for TNF-induced lethal SIRS of GRdim/dim mice was reverted by pretreatment with Nec1s (29), a specific necroptosis inhibitor (Figure 6G). These data combined suggest that necroptosis is a contributing pathway that is more strongly induced by TNF in GRdim/dim mice, potentially because the Ripk3, Zbp1, and Mlkl genes are more strongly induced in these mice.

Discussion

GCs are powerful in protecting against the TNF-induced lethal shock model (7), but the mechanism of protection is not clear. Here, we show that DEX confers protection against TNF in GRWT/WT mice, yet fails to do so in GRdim/dim mice. Both GRdim/dim and GRVillinKO mice exhibit a stronger response to TNF with gut barrier leakiness. DEX failed to provide protection against TNF in both these mutant mice. GRdim/dim mice exhibited more structural damage and cell death of IECs in response to TNF.

RNA-seq analysis on IECs of GRWT/WT and GRdim/dim mice under basal conditions revealed an unexpected and strong IFN signature in GRdim/dim mice. The phosphorylation state of STAT1 in GRdim/dim mice implied that STAT1 is more transcriptionally active. The enhanced expression of Stat1 and ISGs depends on the GR, since the same ISG signature could be seen in IECs of GRVillinKO mice and, importantly, appeared to be IEC specific. Interestingly, the ISG signature in IECs of GRdim/dim mice was confirmed by a proteome-wide MS experiment: 32 proteins were identified as substantially upregulated, including 17 IFN-stimulated proteins, such as STAT1.

The RNA-seq analysis showed that, under basal conditions, 664 genes were DE in GRdim/dim mice; of these, 460 were upregulated and 204 downregulated. This result is in contrast with the genome-wide expression profiling by Frijters et al., in which no DE genes were found in liver samples of GRWT/WT and GRdim/dim mice. Based on their findings, the authors suggested that the GR dim mutation itself does not cause differential gene regulation (13). The discrepancy between their liver and our IEC results might be explained by the fact that local GC production in the gut could lead to GR dimer formation even in the absence of exogenous ligand. It is important to note that recent studies in unchallenged mice suggest that, in the absence of synthetic ligand, the DNA-bound GR almost exclusively resides in its monomer form and that virtually the complete population of GR forms GR dimers upon injection of pharmacological doses of ligand (14). Hence, it is plausible that, in organs where GCs are produced locally, CS levels rise to a level sufficient to support GR dimerization, leading to transactivation or repression of particular GR dimer–sensitive target genes. This mechanism may play a role in the IECs, as these are known to produce CS (19).
Figure 6. Exogenous GC treatment represses ISG signature in a GR dimer–dependent way. (A–E) Analysis of the DEX repression on TNF–induced ISGs based on RNA-seq data. GRWT/WT (black) and GRdim/dim (white) mice were pretreated with vehicle or 10 mg/kg DEX for 30 minutes, followed by challenge with 50 μg or 12.5 μg TNF, respectively (n = 3 per group). RNA was isolated and Stat1 (A) and Ifit1 (B) mRNA expression were analyzed via qPCR. (C) 736 ISGs were induced by TNF in GRWT/WT and GRdim/dim mice. The impact (LFC) of DEX pretreatment is depicted as a percentage of reduction. (D) In total, across GRWT/WT and GRdim/dim mice, 2,029 genes were induced by TNF, and 93 genes were only significantly inhibited by DEX in GRWT/WT mice. (E) Average normalized counts determined by RNA-seq for Ripk3, which is one of these 93 genes. All bars represent mean ± SEM, and statistical analysis was with 2-way ANOVA. (F and G) GRdim/dim mice have an increased necroptosis signature in IECs. GRWT/WT (black) and GRdim/dim mice (white) were injected with PBS or 12.5 μg TNF (n = 5 per group). After 2 hours, Ripk3 was detected on ileum samples by IHC (F; white arrows; representative images are shown). (G) GRdim/dim mice were pretreated with vehicle (black) or 250 μg Nec1s (white). Thirty minutes later, mice were challenged with 12.5 μg TNF, and survival was monitored (n = 5 per group). P values for survival curves were analyzed with a log-rank test. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05. (H) General overview of the interaction between the microbiota and local CS production in controlling the ISG signature in IECs. PRR, pattern recognition receptor; TNFR, TNF receptor.

As an additional explanation for the gut specificity of this ISG signature, the role of the gut microbiota was investigated. Elimination of this factor by antibiotics strongly (but not completely) reduced expression of Stat1 and other ISGs and led to some, albeit incomplete, protection against TNF-induced lethality in GRdim/dim mice. These results suggest a link between ISG signature and TNF sensitivity, which is in line with earlier data using IFN receptor–KO mice (3). The fact that antibiotics only partly protect against TNF and do not completely revert the GRdim sensitivity for TNF suggests that either the elimination of gut flora is incomplete, e.g., leaving certain resistant bacteria, viruses, and fungi intact, or that the contribution of ISGs is only partial. Stat1, however, is a central player in TNF-induced lethal SIRS as well as gut permeability, as we show using Stat1−/− mice. Unfortunately GRdim/dimStat1−/− mice appeared not viable due to an unknown cause. Tofacitinib, a JAK/STAT1 inhibitor, like antibiotics, led to a marked yet incomplete reversal of the TNF sensitivity of GRdim/dim mice.

Our data suggest that endogenous GCs are (a) induced in a gut–flora–dependent way as a result of the ISG signature, which includes the steroidogenic Cyp11a1 and Cyp11b1 genes, but (b) also repress ISGs via GR dimers. Since Stat1 is a central core node regulator of ISGs, it is conceivable that GR dimers block ISGs at the level of Stat1. Activated Stat1/Stat1 homodimers or Stat1/Stat2 heterodimers translocate to the nucleus, where they participate in the regulation of the expression of ISGs (26). GCs are known to modulate IFN signaling at different levels. First, GCs repress type I IFN production, leading to reduced expression of ISGs (30, 31). Second, GCs inhibit Stat1 activation at therapeutic, but also physiological, levels by repressing its mRNA and protein expression (32). Third, Stat1 phosphorylation and occupancy of ISRE sequences in the DNA are actively inhibited by GC treatment (33–35). Inhibition of the Stat1 system, including expression, phosphorylation, and occupancy of ISREs, might be an important part of the antinflammatory capacities of GCs (34). However, we found no such evidence in GRdim/dim mice. Fourth, increased Stat1 expression could result from binding of GR dimers to GRE elements with nGRE activity, leading to downregulation of gene expression. Several GRE elements have recently been described in the mouse Stat1 gene (36). Fifth, and of interest here, GR dimers inhibit Stat1 transcription via so-called IR-nGREs (consensus sequence: CTCC(N)₆GGAGA), first described by Surjit et al., and present in the promoter of many genes, leading to transcriptional repression via binding of GR dimers (15). In silico analysis of the Stat1 promoter predicted 2 IR-nGRE elements (IR-nGRE1 and IR-nGRE2). Although we found no difference in GR occupancy on these elements between naive GRWT/WT and GRdim/dim mice, we clearly found GR recruitment after DEX injection in GRWT/WT mice, but not in GRdim/dim mice, suggesting a dimer-specific interaction. It is also important to note that, although Surjit et al. clearly showed that DEX-induced IR-nGRE-mediated repression was abolished in GRdim/dim mice, it is suggested that GR dimers are formed in a structurally alternative way on these elements (37). Sixth, since the genes that are downregulated by DEX in IECs in a GR dimer–dependent way appear to be strongly enriched in IRF1 and ISRE sequences, GR dimers may bind to IRF1 on the one hand or STAT1, STAT2, or IRF9 on the other hand and form a transcriptionally inhibitory complex.

The genes coding for key enzymes of CS synthesis were found to be upregulated in naive GRdim/dim mice, which is compatible with the fact that they are known in the Interferome database as ISGs (17). Antibiotics downregulate expression of these genes and also downregulate IEC-specific production of CS. These data are in line with the hypothesis that IECs are stimulated by gut flora to produce ISGs, including genes leading to local CS production, which triggers GR dimers to suppress this ISG signature at the level of Stat1. Larsson et al. showed dramatic transcriptional responses to microbiota in all segments of the gut by comparing the gene-expression profile of germ-free and conventionally raised WT mice (38). Similarly to what was shown in other studies (39), Larsson et al. observed a significant microbial induction of genes related to immunity. Moreover, sensing of microbial products by various receptors triggers ISG expression via IFN-β and IFN-α production (22).

IFNs are known to prime cells for subsequent inflammatory triggers (40). By RNA-seq in IECs of GRWT/WT and GRdim/dim mice, we found that TNF induces ISGs and that an equal dose in both groups of mice led to higher LFCs of ISG expression in GRdim/dim mice. IFN signaling and activation of IRF were the 2 most prominent pathways predicted by IPA in GRdim/dim mice injected with a lethal dose of TNF compared with GRWT/WT mice injected with a lethal TNF dose. These data suggest that the increased sensitivity for TNF in GRdim/dim mice is based on an uncontrolled induction of ISGs, not on a priming sensu stricto.

Finally, to understand why GRdim/dim mice are not protected against TNF-induced permeability and lethality by exogenous GCs, we studied the impact of DEX on TNF-induced changes in IECs and performed genome-wide expression profiling via RNA-seq in IECs of DEX- and DEX/TNF-treated GRWT/WT and GRdim/dim mice. DEX inhibits several TNF-induced physiological changes in a dimer–dependent way, namely gut permeability, gut damage, and cell death. The transcription factor–binding motifs found in the genes repressed by DEX in a dimer–specific way were ISRE and IRF elements only. This suggests a poor repression of ISGs in
**Methods**

**Mice.** GRdim/dim mice were generated by H.M. Reichardt et al. and kept on an FVB/N background (11). Heterozygous GRdim/WT mice were intercrossed to generate GRWT/WT and GRdim/dim homozygous mutant mice. All offspring were genotyped by PCR on genomic DNA isolated from toe biopsies. GR8/WT mice (46) were crossed with Villin-Cre transgenic mice (47) and the offspring intercrossed to generate GRVillinKO and GRfl/fl mice, all on an FVB/N background. Heterozygous GRdim/WT mice were intercrossed to generate GRWT/WT, respectively. Mice were kept in individually ventilated cages under a 12-hour dark/12-hour light cycle in a conventional and specific pathogen-free (SPF) animal facility and received food and water ad libitum. All mice were used at 8 to 12 weeks of age.

**Reagents.** Recombinant mouse TNF was produced in *E. coli* and purified to homogeneity in our laboratories. TNF had a specific activity of 1.2 × 10⁸ IU/mg and no detectable endotoxin contamination. DEX (D-4902) and FD4 FITC-dextran (FD4-1G, Mw 3,000–5,000 Da) were purchased from Sigma-Aldrich NV. Recombinant TNF, DEX, and FITC-dextran were diluted in pyrogen-free PBS. Tofacitinib (CP-690550) citrate (S5001; Shelleckchem Inc.) was diluted in 2% Tween 80 with 0.5% methylcellulose diluted in PBS. Nec1s, a necroptosis inhibitor, was diluted in DMSO and was used as recently described (48).

**Injections and sampling.** All injections were given intraperitoneally except in Nec1s experiments, for which intravenous injections were used. Injection volumes were always adapted to the body weight of the mice. In lethality experiments, lethal response was followed until no further deaths occurred. FITC-dextran and tofacitinib were administered via oral gavage. Blood was taken via cardiac puncture after sedation of the mice with a ketamine/xylazine solution (Sigma-Aldrich NV). To obtain mouse serum, samples were allowed to clot overnight at 4°C. The next day, the clot was removed and samples were centrifuged at 20,000 g for 3 minutes. Serum samples were stored at -20°C. For sampling of different organs and IECs, the mice were killed by cervical dislocation at indicated time points. IEC samples for RNA isolations were prepared as follows: ileum was dissected, extensively flushed with PBS, and incubated with lysis buffer (732 6802; Bio-Rad) supplemented with 2-mercaptoethanol on ice for 5 minutes. Samples were then snap-frozen in liquid nitrogen. IEC samples for Western blot analysis were also snap-frozen. For histology, a piece of ileum was fixed in 4% paraformaldehyde and embedded in paraffin by a standard protocol.

**Intestinal permeability.** An in vivo permeability assay was performed using FITC-dextran as described previously (4). Three hours after TNF challenge, 100 µl FITC-dextran (25 mg/ml in PBS) was administered by oral gavage. Five hours later, blood was collected in an EDTA-coated tube and centrifuged at 500 g for 10 minutes. Plasma was collected and fluorescence was measured as follows: λexc/λem = 488/520 nm.

**Tissue sections, histology, and immunohistochemistry.** Tissue sections of 4 µm were cut and stained with H&E using standard protocols. Pictures were taken with an Olympus Bx51 Light microscope (×40). Tissue damage was quantified by 4 neutral observers using the necrotizing enterocolitis scoring system published and validated by Halpern et al. (28). The mean values of all estimations were used. Cell death was identified by TUNEL (Fluorescein In Situ Cell Death Detection Kit; Roche) according to the manufacturer’s protocol.

**Immunohistochemistry of RIPK3.** For immunohistochemistry, tissue sections were dewaxed, incubated in Dako Antigen Retrieval Solution (Agilent) at a boiling temperature for 20 minutes in a Puck cell cooking unit, and cooled down for 2.5 hours. Blocking buffer (5% goat serum in PBT, i.e., PBS, 0.1% BSA, and 1% Triton X-100) was added to the slides for 30 minutes at room temperature. Primary antibody against RIPK3 (ADI-905-242-100; Enzo Life Sciences) was diluted 1:200 in PBT and incubated overnight at 4°C. Slides were then incubated with secondary antibody (goat anti-rabbit-labeled E0432, Dako, Agilent; 1:500 in blocking buffer) and streptavidin–Alexa Fluor 568 (S11223, 1:500 in blocking buffer). Counterstaining was done with Hoechst reagent (Sigma-Aldrich NV, 1:1,000 in PBS). Fluorescence microscopy was performed using a Zeiss Axioscan Z.1.
Table 1. Primer sequences used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
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<tbody>
<tr>
<td>Rpl</td>
<td>CCTGCGCTCCTGAAGGTT</td>
<td>TGGGTACCGCGCTCAATT</td>
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<tr>
<td>Gapdh</td>
<td>TAAGCAGAACTCATCCAGGG</td>
<td>CAAAGCATGGATGGTGGAG</td>
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<tr>
<td>Hprt</td>
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<td>AGCTTTACACTGGATCAAG</td>
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<tr>
<td>Ubc</td>
<td>ACTCTCGAAGCATGCTCCT</td>
<td>TCAACGACCAAGACCAAGA</td>
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<td>TAAAGCCTCTTACAAATCA</td>
<td>ACGACTCAATCAGAACAGG</td>
</tr>
<tr>
<td>Ifβ</td>
<td>AGACACTTCCCTCCTCC</td>
<td>CATATCCTGCTGCGTAGA</td>
</tr>
<tr>
<td>If1</td>
<td>CTGAAATGTCCTAATGCGA</td>
<td>CGTATCTCCGCTGCTCC</td>
</tr>
<tr>
<td>If2</td>
<td>ATGCAAACCCCCAAGCAGC</td>
<td>GATTGACATGTTGCGTAGG</td>
</tr>
<tr>
<td>Stat1</td>
<td>TACACCGGTCGACTGCTTACG</td>
<td>GAAACAGCAAACATGAGAC</td>
</tr>
<tr>
<td>Gyp1α</td>
<td>ATCCTCTTAAGATGATGCTT</td>
<td>TCTCCTGAATGGGGCCATAC</td>
</tr>
<tr>
<td>Gyp1β</td>
<td>AAACCAATGGTCTGCTCAACA</td>
<td>CAAAGTCTCTTTGCTTCCAT</td>
</tr>
</tbody>
</table>

qPCR analysis. RNA was isolated with the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. RNA concentration was measured with the Nanodrop 1000 (Thermo Fisher Scientific), and 300–1,000 ng RNA was used to prepare cDNA with Transcriptor II (Invitrogen, Thermo Fisher Scientific). qPCR was performed using the Roche LightCycler 480 System (Applied Biosystems). The best performing housekeeping genes were determined by geNorm (49). Results are given as relative expression values normalized to the geometric mean of the housekeeping genes. Primers used for qPCR are depicted in Table 1.

RNA-seq analysis of IEC samples. Total RNA was isolated with the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Biological triplicates were used for every condition. RNA concentration was measured, and RNA quality was checked with the Agilent 2100 Bioanalyzer. gDNA contamination was measured, and RNA integrity was checked with the Agilent 2100 Bioanalyzer. Total RNA was isolated with the RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. Biological triplicates were used for every condition. RNA concentration was measured, and RNA quality was checked with the Agilent 2100 Bioanalyzer.

Proteomics sample preparation, LC-MS/MS analysis, and data analysis. See Supplemental Methods.

Western blot analysis. For the detection of STAT1 and p-STAT1, protein was isolated out of IECs (lysis buffer with 0.1% SDS, 150 mM NaCl, 50 mM Tris pH 8, 1% NP-40, 0.5% deoxycholate, 0.5 mM EDTA supplemented with protease and phosphatase inhibitor cocktails from Roche). Protein samples containing 50 µg protein were separated by electrophoresis in a 10% gradient SDS polyacrylamide gel and transferred to nitrocellulose membranes (pore size, 0.45 µm). After blocking the membranes with 1:2 dilution of Starting Block/PBST 0.1%

Antibiotic-mediated depletion of commensal bacteria. Mice were kept in individual cages and treated with a dilution of a 100 mg/ml ciprofloxacin (Sigma-Aldrich NV), 500 mg/ml ampicillin (Sigma-Aldrich NV), 500 mg/l metronidazole (Sigma-Aldrich NV), and 250 mg/l vancomycin (Duchefa Biochemie) solution in their drinking water for 3 weeks. After 2 weeks, the presence of microflora was determined by
culturing fecal samples in brain-heart infusion (BD) and thioglycollate medium (Sigma-Aldrich NV).

**Measuring CS produced by IECs using ileum explants.** Mice were anesthetized with a ketamine/xylazine solution and perfused via the vena cava inferior with PBS supplemented with heparin to remove contamination of peripheral tissues by adrenal-produced CS. After perfusion, ileum samples of equal size were collected and flushed with PBS in order to remove remaining feces. Next, ileum samples were cut longitudinally and into small pieces that were incubated in Opti-MEM at 37°C. Supernatant of ileum explants was collected after 5 hours, and CS levels were determined with the Corticosterone EIA Kit (Arbor Assays) and according to the supplier’s standard protocol.

**Statistics.** For dose curve experiments, 95% confidence intervals were calculated for the LD₅₀ of each group by GenStat, 15th edition. Statistics on survival curves were performed using the log-rank test, but a χ² test was used in order to determine differences between final outcomes. Statistical significance of differences between groups was determined by 2-tailed Student’s t tests, 1-way ANOVA, or 2-way ANOVA tests with 95% confidence intervals. All data, except RNA-seq data, are expressed as mean ± SEM and analyzed using GraphPad Prism software. P < 0.05 was considered significant.

**Study approval.** All experiments described in this paper were approved by the ethical committee of the Faculty of Sciences, Ghent University.

**Author contributions**

MB, KVL, ST, ME, SV, FT, KD, JS, JV, LVW, RDR, NT, FI, and REV performed and guided experimental work. CL supervised the design and performance of the experiments. ST performed the analysis of RNA-seq data and made graphical overviews for the manuscript. ME, KD, and JS provided technical assistance. HMR and JT provided the GR°/°° and GR°/° mice, respectively. RB, PV, FI, and KDB provided other essential tools and useful comments and suggestions. MB and CL wrote the manuscript.

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