

1 **IFN $\beta$  and TNF $\alpha$  cooperate to induce a STAT1-independent antiviral**  
2 **and immunoregulatory program via non-canonical STAT2 and IRF9**  
3 **pathways**  
4

5 **Mélissa K. Mariani<sup>1</sup>, Pouria Dasmeh<sup>2,3</sup>, Audray Fortin<sup>1</sup>, Elise Caron<sup>1</sup>, Mario Kalamujic<sup>1</sup>,**  
6 **Alexander N. Harrison<sup>1,4</sup>, Dacquin M. Kasumba<sup>1,2</sup>, Sandra Cervantes-Ortiz<sup>1,5</sup>, Espérance**  
7 **Mukawera<sup>1</sup>, Adrian W.R. Serohijos<sup>2,3</sup> and Nathalie Grandvaux<sup>1,2\*</sup>**

8  
9 <sup>1</sup> CRCHUM - Centre Hospitalier de l'Université de Montréal, Québec, Canada

10 <sup>2</sup> Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Université de Montréal,  
11 Qc, Canada.

12 <sup>3</sup> Centre Robert Cedergren en Bioinformatique et Génomique, Université de Montréal, Qc, Canada

13 <sup>4</sup> Department of Microbiology and Immunology, McGill University, Qc, Canada

14 <sup>5</sup> Department of Microbiology, Infectiology and Immunology, Faculty of Medicine, Université de  
15 Montréal, Qc, Canada

16  
17 **\* Correspondence:**

18 Corresponding Author

19 [nathalie.grandvaux@umontreal.ca](mailto:nathalie.grandvaux@umontreal.ca)

20  
21 **Keywords:** Interferon  $\beta$ , TNF $\alpha$ , STAT1, STAT2, IRF9, antiviral,

22 **Running title:** IFN $\beta$ +TNF $\alpha$ -mediated STAT1-independent transcriptional program

1 **ABSTRACT**

2 IFN $\beta$  typically induces an antiviral and immunoregulatory transcriptional program through the  
3 activation of ISGF3 (STAT1, STAT2 and IRF9) transcriptional complexes. The response to IFN $\beta$  is  
4 context-dependent and is prone to crosstalk with other cytokines, such as TNF $\alpha$ . IFN $\beta$  and TNF $\alpha$   
5 synergize to drive a specific delayed transcriptional program. Previous observation led to the  
6 hypothesis that an alternative STAT1-independent pathway involving STAT2 and IRF9 might be  
7 involved in gene induction by the combination of IFN $\beta$  and TNF $\alpha$ . Using genome wide  
8 transcriptional profiling by RNASeq, we found that the costimulation with IFN $\beta$  and TNF $\alpha$  induces a  
9 broad antiviral and immunoregulatory transcriptional program independently of STAT1.  
10 Additionally, STAT2 and IRF9 are involved in the regulation of only a subset of these STAT1-  
11 independent genes. Consistent with the growing literature, STAT2 and IRF9 act in concert to regulate  
12 a subgroup of these genes. Unexpectedly, STAT2 and IRF9 were also engaged in specific  
13 independent pathways to regulate distinct sets of IFN $\beta$  and TNF $\alpha$ -induced genes. Altogether these  
14 observations highlight the existence of distinct previously unrecognized non-canonical STAT1-  
15 independent, but STAT2 and/or IRF9-dependent pathways in the establishment of a delayed antiviral  
16 and immunoregulatory transcriptional program in conditions where elevated levels of both IFN $\beta$  and  
17 TNF $\alpha$  are present.

18

## 1 INTRODUCTION

2 Interferon (IFN)  $\beta$  plays a critical role in the first line of defense against pathogens,  
3 particularly viruses, through its ability to induce a broad antiviral transcriptional response in virtually  
4 all cell types<sup>1</sup>. IFN $\beta$  also possesses key immunoregulatory functions that determine the outcome of  
5 the adaptive immune response against pathogens<sup>1, 2</sup>. IFN $\beta$  acts through binding to the IFNAR  
6 receptor (IFNAR1 and IFNAR2) leading to Janus kinases (JAK), JAK1 and Tyk2, mediated  
7 phosphorylation of signal transducer and activator of transcription (STAT) 1 and STAT2, and to a  
8 lesser extent other STAT members in a cell-specific manner<sup>3, 4</sup>. Phosphorylated STAT1 and STAT2  
9 together with IFN Regulatory Factor (IRF) 9 form the IFN-stimulated gene factor 3 (ISGF3) complex  
10 that binds to the consensus IFN-stimulated response element (ISRE) sequences in the promoter of  
11 hundreds of IFN stimulated genes (ISGs)<sup>5</sup>. Formation of the ISGF3 complex is considered a  
12 hallmark of the engagement of the type I IFN response, and consequently the requirement of STAT1  
13 in a specific setting has become a marker of the engagement of type I IFN signaling<sup>3, 6</sup>. However, in  
14 recent years this paradigm has started to be challenged with accumulating evidence demonstrating  
15 the existence of non-canonical JAK-STAT signaling mediating type I IFN responses<sup>4, 7</sup>.

16  
17 Over the years, *in vitro* and *in vivo* studies aimed at characterizing the mechanisms and the  
18 functional outcomes of IFN $\beta$  signaling were mostly performed in relation to single cytokine  
19 stimulation. However, this unlikely reflects physiological settings, as a plethora of cytokines is  
20 secreted in a specific situation. As a consequence, a cell rather simultaneously responds to a cocktail  
21 of cytokines to foster the appropriate transcriptional program. Response to IFN $\beta$  is no exception and  
22 is very context-dependent, particularly regarding the potential cross-talk with other cytokines. IFN $\beta$   
23 and TNF $\alpha$  exhibit context-dependent cross-regulation, but elevated levels of both cytokines are  
24 found during the host response to pathogens, including virus and bacteria, and also in

1 autoinflammatory and autoimmune diseases<sup>8</sup>. While the cross-regulation of IFN $\beta$  and TNF $\alpha$  is well  
2 studied, the functional cross-talk between these two cytokines remains poorly known and is limited  
3 to the description of a synergistic interaction<sup>9-12</sup>. Indeed, costimulation with IFN $\beta$  and TNF $\alpha$  was  
4 found to drive a specific delayed transcriptional program composed of genes that are either not  
5 responsive to IFN $\beta$  or TNF $\alpha$  separately or are only responsive to either one of the cytokine<sup>10,11</sup>.

6  
7 The signaling mechanisms engaged downstream of the costimulation with IFN $\beta$  and TNF $\alpha$   
8 remained elusive, but it is implicitly assumed that the fate of the gene expression response requires  
9 that both IFN $\beta$ -and TNF $\alpha$ -induced signaling pathways exhibit significant cross-talk. Analysis of the  
10 enrichment of specific transcription factors binding sites in the promoters of a panel of genes  
11 synergistically induced by IFN $\beta$  and TNF $\alpha$  failed to give a clue about the specificity of the  
12 transcriptional regulation of these genes<sup>10</sup>. Recently, analysis of the induction of *DUOX2* and  
13 *DUOXA2* genes, which belong to the category of delayed genes that are remarkably induced to high  
14 levels in response to the combination of IFN $\beta$  and TNF $\alpha$ , led to the hypothesis that STAT2 and IRF9  
15 activities might segregate in an alternative STAT1-independent pathway that could be involved in  
16 gene induction downstream of IFN $\beta$  and TNF $\alpha$ <sup>12</sup>. Further validation was awaited to confirm the  
17 existence of this STAT1-independent response and the extent to which it is involved in the regulation  
18 of a specific delayed transcriptional program induced by the combination of IFN $\beta$  and TNF $\alpha$ .

19  
20 In the present study, we aimed to characterize the transcriptional profile of the delayed  
21 response to IFN $\beta$  and TNF $\alpha$  in the absence of STAT1 and evaluate the role of STAT2 and IRF9 in  
22 the regulation of this response. Taking advantage of STAT1-deficient cells, we found that the  
23 synergistic action of IFN $\beta$  and TNF $\alpha$  induces a broad delayed antiviral and immunoregulatory  
24 transcriptional program independently of STAT1. We also report that STAT2 and IRF9 are

1 differentially involved in the regulation of distinct subsets of genes induced by IFN $\beta$  and TNF $\alpha$   
2 While IFN $\beta$  and TNF $\alpha$  act in part through the concerted action of STAT2 and IRF9, specific sets of  
3 genes were only regulated either by STAT2 or IRF9. These findings highlight the existence of  
4 distinct previously unrecognized non-canonical STAT2 and/or IRF9-dependent pathways that  
5 mediate the synergistic action of IFN $\beta$  and TNF $\alpha$ .

6

## 7 **RESULTS**

8

### 9 **Distinct induction profiles of antiviral and immunoregulatory genes in response to IFN $\beta$ , TNF $\alpha$** 10 **and IFN $\beta$ +TNF $\alpha$ .**

11 First, we sought to determine the induction profile of a selected panel of immunoregulatory  
12 and antiviral genes in response to IFN $\beta$ +TNF $\alpha$  in comparison to IFN $\beta$  or TNF $\alpha$  alone. A549 cells  
13 were stimulated either with IFN $\beta$ , TNF $\alpha$  or IFN $\beta$ +TNF $\alpha$  for various times between 3-24h and the  
14 relative mRNA expression levels were quantified by qRT-PCR. Analysis of the expression of the  
15 selected genes revealed distinct profiles of response to IFN $\beta$ , TNF $\alpha$  or IFN $\beta$ +TNF $\alpha$  (**Figure 1**).  
16 *IDO*, *DUOX2*, *CXCL10*, *APOBEC3G*, *ISG20* and *IL33* exhibited synergistic induction in response to  
17 IFN $\beta$ +TNF $\alpha$  compared to IFN $\beta$  or TNF $\alpha$  alone. Expression in response to IFN $\beta$ +TNF $\alpha$  increased  
18 over time, with maximum expression levels observed between 16 and 24h. While *NOD2* and *IRF1*  
19 induction following stimulation with IFN $\beta$ +TNF $\alpha$  was also significantly higher than upon IFN $\beta$  or  
20 TNF $\alpha$  single cytokine stimulation, they exhibited a steady-state induction profile starting as early as  
21 3h. *MX1* and *PKR*, two typical IFN $\beta$ -inducible ISGs, were found induced by IFN $\beta$ +TNF $\alpha$  similarly  
22 to IFN $\beta$  alone and were not responsive to TNF $\alpha$ . *CCL20* responded to IFN $\beta$ +TNF $\alpha$  with a kinetic  
23 and amplitude similar to TNF $\alpha$ , but was not responsive to IFN $\beta$  alone. *IL8* expression was not

1 induced by IFN $\beta$ , but was increased by TNF $\alpha$  starting at 3h and remained steady until 24h. In  
2 contrast to other genes, *IL8* induction in response to IFN $\beta$ +TNF $\alpha$  was significantly decreased  
3 compared to TNF $\alpha$  alone. Overall, these results confirm that induction of a subset of antiviral and  
4 immunoregulatory genes is greatly increased in response to IFN $\beta$ +TNF $\alpha$  compared to either IFN $\beta$  or  
5 TNF $\alpha$  alone.

6

### 7 **Workflow for genome-wide characterization of the delayed transcriptional program induced** 8 **by IFN $\beta$ +TNF $\alpha$ in the absence of STAT1.**

9 In a previous report, we provided evidence of a STAT1-independent, but STAT2- and IRF9-  
10 dependent, pathway engaged downstream of IFN $\beta$ +TNF $\alpha$  <sup>12</sup>. Here, taking advantage of the STAT1-  
11 deficient human U3A cell line <sup>13</sup>, we aimed to fully characterize the STAT1-independent  
12 transcriptional program induced by IFN $\beta$ +TNF $\alpha$ . Two hallmarks of STAT2 and IRF9 activation, *i.e.*  
13 STAT2 Tyr690 phosphorylation and induction of IRF9, were observed in U3A cells following  
14 stimulation with IFN $\beta$ +TNF $\alpha$ . Although STAT2 and IRF9 activation was reduced compared to the  
15 parental 2ftGH cells expressing endogenous STAT1, this observation supports the capacity of  
16 STAT2 and IRF9 to be activated in STAT1-deficient U3A cells stimulated with IFN $\beta$ +TNF $\alpha$ .  
17 **(Figure 2A).**

18

19 To investigate the transcriptional program triggered independently of STAT1, the U3A cells  
20 were efficiently transfected with Control (Ctrl)-, STAT2- or IRF9-RNAi and further left untreated or  
21 stimulated with IFN $\beta$ +TNF $\alpha$  for 16h **(Figure 2B)**. Efficient silencing was confirmed by immunoblot  
22 **(Figure 2C)**. To perform genome-wide transcriptional analysis, total RNA was isolated and analyzed  
23 by RNA sequencing (n=3 for each group detailed in **Figure 2B**) on an Illumina HiSeq2500 platform.  
24 To validate the RNASeq data, the fold changes (FC) of 13 genes between the different experimental

1 groups, siCTRL non-stimulated (NS) *vs* siCTRL IFN $\beta$ +TNF $\alpha$ , siCTRL IFN $\beta$ +TNF $\alpha$  *vs* siSTAT2  
2 IFN $\beta$ +TNF $\alpha$  and siCTRL IFN $\beta$ +TNF $\alpha$  *vs* siIRF9 IFN $\beta$ +TNF $\alpha$  was confirmed by qRT-PCR. A  
3 positive linear relationship between RNASeq and qRT-PCR FC was observed (**Figure 2D**).

4

## 5 **An antiviral and immunoregulatory transcriptional signature is induced by IFN $\beta$ +TNF $\alpha$** 6 **independently of STAT1.**

7 To identify STAT1-independent differentially expressed genes (DEGs) upon IFN $\beta$ +TNF $\alpha$   
8 stimulation, comparison between non-stimulated (NS) and IFN $\beta$ +TNF $\alpha$  stimulated control cells was  
9 performed (**Figure 2E**). In total, 612 transcripts, including protein-coding transcripts, pseudogenes  
10 and long non-coding RNA (lncRNA), were significantly different (FC>1.5, p<0.05, FDR<0.05) in  
11 IFN $\beta$ +TNF $\alpha$  *vs* NS. Among these, 482 DEGs were upregulated and 130 were downregulated  
12 (**Figure 3A**; See **Supplemental Table S1** for a complete list of DEGs). To identify the Biological  
13 Processes (BP) and Molecular Functions (MF) induced by IFN $\beta$ +TNF $\alpha$  independently of STAT1, we  
14 first subjected upregulated DEGs through Gene Ontology (GO) enrichment analysis. The top  
15 enriched GO BP (p< 10<sup>-10</sup>) and MF, are depicted in **Figure 3B** (See **Supplemental Table S2** for a  
16 complete list of enriched GO). The majority of the top enriched BPs were related to cytokine  
17 production and function (response to cytokine, cytokine-mediated signaling pathway, cytokine  
18 production, regulation of cytokine production), immunoregulation (Immune response, immune  
19 system process, innate immune response, regulation of immune system process) and host defense  
20 response (defense response, response to other organism, 2'-5'-oligoadenylate synthetase activity and  
21 dsRNA binding). Fourteen MF categories were found enriched in IFN $\beta$ +TNF $\alpha$ . The top enriched  
22 MFs were related to cytokine and chemokine functions (cytokine activity, cytokine receptor binding,  
23 chemokine activity, Interleukin 1-receptor binding). Other enriched MF included peptidase related  
24 functions (endopeptidase inhibitor activity, peptidase regulator activity, serine-type endopeptidase

1 activity).

2

3 To further gain insight into the functional significance of the STAT1-independent  
4 IFN $\beta$ +TNF $\alpha$ -induced transcriptional response, we conducted a modular transcription analysis of  
5 upregulated DEGs against 606 immune-related functional modules. These modules were previously  
6 defined from co-clustered gene sets built via an unbiased data-driven approach as detailed in the  
7 material and methods section <sup>14, 15</sup>. STAT1-independent IFN $\beta$ +TNF $\alpha$ -induced DEGs showed  
8 significant enrichment in 37 modules (**Supplemental Table S3**). Six of these modules were  
9 associated with virus sensing/Interferon antiviral response, including LIM75 (antiviral IFN  
10 signature), LIM68 (RIG-I-like receptor signaling), LIM127 (type I interferon response), LIM111.0  
11 and LIM111.1 (IRF2 target network) and LIM150 (innate antiviral response) (**Figure 3C**).  
12 Additionally, 6 modules were associated with immunoregulatory functions, including LIM29  
13 (proinflammatory cytokines and chemokines), LIM27.0 and LIM27.1 (chemokine cluster I and II),  
14 LIM38 (chemokines and receptors), LIM115 (cytokines receptors cluster) and LIM37.0 (immune  
15 activation - generic cluster) (**Figure 3C**). Module analysis also showed enriched AP-1 transcription  
16 factor-related network modules, LIM20 and LIM0, and cell cycle and growth arrest LIM31  
17 module. To confirm the capacity of IFN $\beta$ +TNF $\alpha$  to trigger an antiviral response independently of  
18 STAT1, U3A cells were stimulated with IFN $\beta$  or IFN $\beta$ +TNF $\alpha$  and further infected with Vesicular  
19 stomatitis Virus (VSV). While VSV replicated similarly in untreated U3A cells and in cells treated  
20 with IFN $\beta$ , treatment with IFN $\beta$ +TNF $\alpha$  significantly restricted VSV replication (**Figure 3D**).  
21 Importantly, IFN $\beta$  treatment led to a significant antiviral response when STAT1 expression was  
22 restored in U3A cells (U3A-STAT1). In this context, the combination of IFN $\beta$ +TNF $\alpha$  was more  
23 effective in restricting VSV than IFN $\beta$  alone (**Figure 3D**). Collectively, these data demonstrate that  
24 the co-stimulation with IFN $\beta$  and TNF $\alpha$  induces a broad antiviral and immunoregulatory



1 transcriptional program that is independent of STAT1.

2

3 **Clustering of STAT1-independent IFN $\beta$ +TNF $\alpha$  induced DEGs according to their regulation by**  
4 **STAT2 and IRF9.**

5 Next, we sought to assess how the STAT1-independent IFN $\beta$ +TNF $\alpha$ -induced antiviral and  
6 immunoregulatory transcriptional response was regulated by STAT2 and IRF9. To do so, we  
7 compared transcripts levels in siSTAT2\_IFN $\beta$ +TNF $\alpha$  vs siCTRL\_IFN $\beta$ +TNF $\alpha$  and  
8 siIRF9\_IFN $\beta$ +TNF $\alpha$  vs siCTRL\_IFN $\beta$ +TNF $\alpha$  conditions (**Figure 2E** and **Supplemental Table S1**).  
9 Volcano plots revealed that a fraction of IFN $\beta$ +TNF $\alpha$ -induced DEGs were significantly (FC>1.5,  
10 p<0.05, FDR<0.05) downregulated or upregulated upon silencing of STAT2 (**Figure 4A**) or IRF9  
11 (**Figure 4B**). Nine distinct theoretical categories of DEGs can be defined based on their potential  
12 individual behavior across siSTAT2 and siIRF9 groups (Categories A-I, **Figure 2E**): a gene can  
13 either be downregulated upon STAT2 and IRF9 silencing, indicative of a positive regulation by  
14 STAT2 and IRF9 (Categorie A); conversely, a gene negatively regulated by STAT2 and IRF9 would  
15 exhibit upregulation upon STAT2 and IRF9 silencing (Categorie B); Genes that do not exhibit  
16 significant differential expression in siSTAT2 and siIRF9 groups would be classified as STAT2 and  
17 IRF9 independent (Categorie C); IRF9-independent genes could exhibit positive (Categorie D) or  
18 negative (Categorie E) regulation by STAT2; conversely, STAT2-independent genes might be  
19 positively (Categorie F) or negatively (Category G) regulated by IRF9; lastly, STAT2 and IRF9  
20 could have opposite effects on a specific gene regulation (Categorie H and I). Based on *a priori*  
21 clustering of RNASeq data (**Figure 4C**) and analysis of the expression of 18 genes by qRT-PCR  
22 (**Figure 4D**), we found that STAT1-independent IFN $\beta$ +TNF $\alpha$ -induced DEGs clustered into only 7 of  
23 the 9 possible categories. Amongst the 482 DEGs, 163 genes exhibited either inhibition or  
24 upregulation following silencing of STAT2 and/or IRF9 (Category B-G). A large majority of

1 upregulated DEGs, i.e. 319 out of the 482 DEGs, were not significantly affected by either STAT2 or  
2 IRF9 silencing, and were therefore classified as STAT2/IRF9-independent (**Figure 4C**). No genes  
3 were found in categorie H and only one gene was found in categorie I.

4  
5 To functionally interpret these clusters, we applied the modular transcription analysis to each  
6 of the categories to assess for the specific enrichment of the functional modules found associated  
7 with IFN $\beta$ +TNF $\alpha$ -upregulated DEGs (**Figure 4E**). First, most modules, except LI.M31 (cell cycle  
8 and growth arrest), LI.M38 (chemokines and receptors), LI.M37.0 (immune activation - generic  
9 cluster) and LI.M53 (inflammasome receptors and signaling), were enriched in the category of DEGs  
10 positively regulated by STAT2 and IRF9 (Category A). Conversely, the cluster negatively regulated  
11 by STAT2 and IRF9 (Category B) exclusively contains enriched LI.M38 (chemokines and receptors),  
12 LI.M37.0 (immune activation - generic cluster) and LI.M115 (cytokines receptors cluster). The  
13 cluster of IRF9-independent genes that are negatively regulated by STAT2 (Category E) only  
14 exhibited enrichment in the virus sensing/ IRF2 target network LI.M111.0 module, while the IRF9-  
15 independent/ STAT2-positively regulated cluster (Category D) encompasses antiviral and  
16 immunoregulatory functions. The STAT2-independent, but IRF9 positively regulated transcripts  
17 (Category F) was mainly enriched in modules associated with the IFN antiviral response, including  
18 LI.M75 (antiviral IFN signature), LI.M68 (RIG-I-like receptor signaling), LI.M127 (type I interferon  
19 response), and LI.M150 (innate antiviral response). Finally, the STAT2-independent, but IRF9  
20 negatively regulated cluster (Category G) was mostly enriched in modules associated with  
21 immunoregulatory functions, including LI.M29 (proinflammatory cytokines and chemokines),  
22 LI.M27.0 and LI.M27.1 (chemokine cluster I and II), LI.M78 (myeloid cell cytokines), but also with  
23 cell cycle and growth arrest (LI.M31) and inflammasome receptors and signaling (LI.M53). Of note,  
24 all modules were found enriched in the cluster of genes induced independently of STAT2 and IRF9

1 (Category C), pointing to a broad function of this yet to be defined pathway(s) in the regulation of the  
2 antiviral and immunoregulatory program elicited by IFN $\beta$ +TNF $\alpha$ . Altogether these observations  
3 reveal that STAT2 and IRF9 are involved in the regulation of only a subset of the genes induced in  
4 response to the co-stimulation by IFN $\beta$  and TNF $\alpha$  in the absence of STAT1. Importantly, our results  
5 also reveal that STAT2 and IRF9 act in a concerted manner to regulate a specific subset of the  
6 IFN $\beta$ +TNF $\alpha$ -induced DEGs, but are also independently involved in distinct non-canonical pathways.

7

#### 8 **Differential regulation of *CXCL10* in response to IFN $\beta$ and IFN $\beta$ +TNF $\alpha$ .**

9 Identification of DEGs upregulated by IFN $\beta$ +TNF $\alpha$  in a STAT1-independent, but STAT2 and  
10 IRF9-dependent, manner potentially reflects the regulation by an alternative STAT2/IRF9-containing  
11 complex<sup>4, 7</sup>. Whether this STAT2/IRF9 pathway ultimately leads to gene regulation through the  
12 same ISRE sites used by the ISGF3 complex remained to be assessed. In our RNASeq analysis  
13 (**Supplemental Table S1**) and qRT-PCR validation (**Figure 4D**), *CXCL10* belongs to the  
14 IFN $\beta$ +TNF $\alpha$ -induced DEGs that are dependent on STAT2 and IRF9. The *CXCL10* promoter contains  
15 3 ISRE sites. We used full-length wild-type (972bp containing the 3 ISRE sites), truncated (376bp  
16 containing only the ISRE(3) site) or mutated (972bp containing a mutated ISRE(3) site) *CXCL10*  
17 promoter luciferase (*CXCL10*prom-Luc) reporter constructs (**Figure 5A**) to determine the ISRE  
18 consensus site(s) requirement. U3A and STAT1-rescued U3A-STAT1 cells were transfected with the  
19 *CXCL10*prom-Luc constructs and further stimulated with IFN $\beta$  or IFN $\beta$ +TNF $\alpha$  to monitor the  
20 canonical ISGF3-dependent and the STAT1-independent responses. In the absence of STAT1, IFN $\beta$   
21 failed to activate *CXCL10*prom. However, the induction of the *CXCL10*prom activity was restored  
22 either upon expression of STAT1 or when TNF $\alpha$  was used together with IFN $\beta$  (**Figure 5B**). While  
23 the activation of *CXCL10*prom in response to IFN $\beta$  in the presence of STAT1 involves the distal  
24 ISRE(1) and/or ISRE(2) sites and the proximal ISRE(3) site, only the ISRE(3) site is required for

1 induction by IFN $\beta$ +TNF $\alpha$  in the absence of STAT1. Additionally, we also assessed the contribution  
2 of the two NF- $\kappa$ B and the AP-1 sites present in the promoter using *CXCL10*prom-Luc mutated  
3 constructs in comparison to the wild-type reporter. While none of the NF- $\kappa$ B and the AP-1 sites were  
4 required for induction of the *CXCL10* promoter by IFN $\beta$  in the presence of STAT1, the two NF- $\kappa$ B  
5 sites were necessary for the STAT1-independent induction in response to IFN $\beta$ +TNF $\alpha$  (**Figure 5B**).  
6 These observations suggest that the ISRE site usage is more restricted in the absence of STAT1 in the  
7 context of the co-stimulation with IFN $\beta$  TNF $\alpha$  than in the context of an ISGF3-dependent regulation.

8

## 9 **DISCUSSION**

10

11 Our study was designed to determine the functional relevance of a STAT1-independent, but  
12 STAT2- and IRF9-dependent, signaling pathway in the transcriptional program induced by IFN $\beta$  in  
13 the presence of TNF $\alpha$ . Previous studies reported that IFN $\beta$  and TNF $\alpha$  synergize to induce a specific  
14 delayed antiviral program that differs from the response induced by IFN $\beta$  only. This specific  
15 synergy-dependent antiviral response is required for a complete abrogation of Myxoma virus in  
16 fibroblasts <sup>10</sup> and contributes to the establishment of a sustained type I and type III IFNs response  
17 during paramyxovirus infection in airway epithelial cells <sup>12</sup>. The underlying mechanisms of this  
18 specific response remain ill defined, but their elucidation is of particular importance with regards to  
19 conditions with elevated levels of both IFN $\beta$  and TNF $\alpha$  such as pathogen intrusion or, autoimmune  
20 or chronic inflammatory diseases <sup>8</sup>.

21

22 First, we confirmed the previously documented synergistic induction of an IFN $\beta$ +TNF $\alpha$ -  
23 mediated delayed transcriptional program composed of genes that are either not responsive to IFN $\beta$   
24 or TNF $\alpha$  separately or are only responsive to either one of the cytokine when used separately albeit

1 to a lesser extent (**Figure 1**). Using genome wide RNA sequencing, we demonstrate that  
2 IFN $\beta$ +TNF $\alpha$  induces a broad transcriptional program in cells deficient in STAT1. GO enrichment  
3 and transcriptional module analyses showed that STAT1-independent upregulated DEGs encompass  
4 a wide range of immunoregulatory and host defense, mainly antiviral, functions. The STAT1-  
5 independent antiviral response mounted by STAT1-deficient cells in response to IFN $\beta$ +TNF $\alpha$   
6 efficiently restricted VSV replication (**Figure 2**). These findings highlight the functional significance  
7 of a STAT1-independent response.

8

9 We also focused on deciphering the role of STAT2 and IRF9 in the STAT1-independent  
10 transcriptional program elicited in response to IFN $\beta$ +TNF $\alpha$ . We previously found that IFN $\beta$ +TNF $\alpha$   
11 induces the *DUOX2* gene via a STAT2- and IRF9-dependent pathway in the absence of STAT1<sup>12</sup>.  
12 To what extent this pathway contributes to the STAT1-independent response engaged downstream of  
13 IFN $\beta$ +TNF $\alpha$  remained to be addressed. Based on the possible regulation by STAT2 and/or IRF9,  
14 IFN $\beta$ +TNF $\alpha$ -induced DEGs could theoretically be partitioned into 9 different predicted categories  
15 (**Figure 2E**), but we found that they in fact only significantly segregate into 7 categories.  
16 Importantly, the distribution of DEGs amongst 7 different categories reflects distinct contributions of  
17 STAT2 or IRF9 and highlights the heterogeneity of the mechanisms of regulation of the  
18 IFN $\beta$ +TNF $\alpha$ -induced genes. Importantly, only one anecdotic gene was found in categories implying  
19 inverse regulation by STAT2 and IRF9 (categories H and I) pointing to convergent functions of  
20 STAT2 and IRF9 when both are engaged in gene regulation. We can rule out that the distinct  
21 regulation mechanisms reflect distinct profiles of induction by IFN $\beta$ +TNF $\alpha$ . For instance *CXCL10*,  
22 *IL33*, *CCL20* and *ISG20* all exhibit synergistic induction by IFN $\beta$ +TNF $\alpha$  (**Figure 1**), but are  
23 differentially regulated by STAT2 and/or IRF9; while *CXCL10* is dependent on STAT2 and IRF9,

1 *IL33* is independent on STAT2 and IRF9, and *CCL20* and *ISG20* are STAT2-independent but IRF9-  
2 dependent (**Figure 4D**).

3  
4 Consistent with our previous report <sup>12</sup>, we found several genes positively regulated by a non-  
5 canonical STAT2- and IRF9-dependent, but STAT1-independent, pathway (Category A). DEGs in  
6 this category encompass most of the functions induced in response to IFN $\beta$ +TNF $\alpha$ , with the notable  
7 exception of cell cycle and growth arrest and inflammasome and receptor signaling functions. Genes  
8 negatively regulated by STAT2 and IRF9 were also identified (Category B). Accumulating evidence  
9 point to the formation of an alternative STAT2/IRF9-containing complex mediating gene expression  
10 in the absence of STAT1 <sup>16-20</sup>. The specificity of the DNA-binding of a STAT2/IRF9 complex  
11 compared to the ISGF3 complex remains unclear. It was originally found that STAT2/IRF9 exhibit  
12 only limited DNA-binding affinity for the typical ISRE sequence in the absence of STAT1 <sup>16</sup>, but  
13 association of STAT2 with the promoter of antiviral genes induced by Dengue virus in STAT1-  
14 deficient mice was demonstrated by Chromatin immunoprecipitation <sup>21</sup>. Here, the *CXCL10* gene was  
15 further analyzed as a paradigm of STAT2- and IRF9-positively regulated gene. Promoter activity  
16 analysis unveiled distinct ISRE site usage between IFN $\beta$  and IFN $\beta$ +TNF $\alpha$  stimulation (**Figure 5**).  
17 Interestingly, the ISRE site used in response to IFN $\beta$ +TNF $\alpha$  lies close to the NF- $\kappa$ B sites that are  
18 also specifically engaged in this response. A possible mechanism for the synergistic action of  
19 IFN $\beta$ +TNF $\alpha$  might be related to the previous description of the cooperativity between ISGF3 and  
20 NF- $\kappa$ B in the context of *Listeria* infection <sup>22, 23</sup>. In this context, ISGF3 and NF- $\kappa$ B tether a complete  
21 functional mediator multi-subunit complex that bridges transcription factors with Pol II and initiation  
22 and elongation factors to the promoter of antimicrobial genes <sup>22</sup>. Both STAT1 and STAT2  
23 functionally and physically interact with the mediator <sup>24, 25</sup>. Additionally, IL-6 induction by NF- $\kappa$ B  
24 inducers was found synergistically enhanced by IFN $\beta$ . In this model, unphosphorylated STAT2 was

1 proposed to act as a bridge connecting p65 and IRF9 bound to  $\kappa$ B and ISRE consensus sites,  
2 respectively<sup>26</sup>. Therefore, in the absence of STAT1, the synergistic induction of some IFN $\beta$ +TNF $\alpha$ -  
3 induced DEGs might be explained by the interaction between a STAT2/IRF9-containing complex  
4 with NF- $\kappa$ B. However, we cannot exclude that interaction with a yet to be identified alternative  
5 transcription factor might tether STAT2 and IRF9 to the DNA in the absence of STAT1. Specific  
6 regulation of STAT2 and IRF9 might also contribute to the synergistic response elicited by  
7 IFN $\beta$ +TNF $\alpha$ . In a previous study, we observed IRF9 induction and enhanced/extended STAT2  
8 phosphorylation in response to IFN $\beta$ +TNF $\alpha$ <sup>12</sup>. We hypothesized that these events likely contribute  
9 to the specific activation of the non-canonical STAT2/IRF9-dependent pathway. Remarkably, a  
10 similar prolonged STAT2 phosphorylation was observed upon stimulation of STAT1 KO bone  
11 marrow-derived murine macrophages with IFN $\alpha$ . In this context, a STAT2/IRF9 complex was  
12 shown to induce a delayed set of ISGs<sup>20</sup>. Taken together, it is reasonable to speculate that in a  
13 physiological context, when TNF $\alpha$  is present with IFN $\beta$ , a signal is elicited to progressively exclude  
14 STAT1 from the STAT2/IRF9 complex and favor the non-canonical STAT2/IRF9 pathway to drive a  
15 specific delayed response.

16

17 The observation that some IFN $\beta$ +TNF $\alpha$ -induced genes were independent of IRF9 but  
18 dependent on STAT2, either positively or negatively (Categories D and E), in a STAT1 deficient  
19 context might reflect the previous observation that STAT2 forms alternative complexes with other  
20 STAT members. STAT2 was shown to associate with STAT3 and STAT6, although it is not  
21 completely clear whether IRF9 is also required in these alternative complexes<sup>27, 28</sup>. Interestingly,  
22 transcriptional module analyses demonstrated that the functional distribution of genes negatively  
23 regulated by STAT2 is very limited compared to other categories; only a virus-sensing module was  
24 enriched in this category. In contrary, IRF9-independent genes positively regulated by STAT2

1 mediate broader antiviral and immunoregulatory functions.

2 ISGF3-independent functions of IRF9 have been proposed based on the study of IRF9  
3 deficiencies (reviewed in <sup>7, 29</sup>). However, IRF9 target genes in these contexts have been barely  
4 documented. Intriguingly, Li et al. <sup>30</sup> studied IFN $\alpha$ -induced genes and their dependency on the  
5 ISGF3 subunits. While they confirmed previous studies showing that IFN $\alpha$  can trigger a delayed and  
6 sustained ISGs response via an ISGF3-independent pathway, it is very striking that they did not find  
7 STAT1- and STAT2-independent but IRF9-dependent genes. Indeed, all identified IRF9-dependent  
8 genes were either STAT2- or STAT1-dependent. This result greatly differs with our study. Here, we  
9 found several IFN $\beta$ +TNF $\alpha$ -induced DEGs independent of STAT1 and STAT2, but positively or  
10 negatively regulated by IRF9 (Categories F and G). Typically, IRF9 is considered a positive regulator  
11 of gene transcription. However, our findings are consistent with recent reports documenting the role  
12 of IRF9 in the negative regulation of the TRIF/NF- $\kappa$ B transcriptional response <sup>31</sup> or the expression of  
13 SIRT1 in acute myeloid leukemia cells <sup>32</sup>. The molecular mechanism underlying gene regulation by  
14 IRF9 without association with either STAT1 or STAT2 remain to be elucidated. To the best of our  
15 knowledge, no alternative IRF9-containing complex has been described.

16  
17 Unexpectedly, a vast majority of genes were found to be independent of STAT2 and IRF9  
18 (Category C). All transcriptional modules were enriched in this category pointing to a major role of a  
19 yet to be identified pathway in the establishment of a host defense and immunoregulatory response.  
20 The NF- $\kappa$ B pathway is widely known to be engaged downstream of the TNF receptor. While NF- $\kappa$ B  
21 is an obvious candidate for being involved in the regulation of the STAT2 and IRF9-independent  
22 DEGs, this might fall short in explaining the synergistic action of IFN $\beta$ +TNF $\alpha$ . Synergistic  
23 activation of NF- $\kappa$ B was reported in the context of IFN $\gamma$  and TNF $\alpha$  treatment <sup>33</sup>. However, we did



1 not observe enhanced NF- $\kappa$ B activation upon IFN $\beta$ +TNF $\alpha$  stimulation compared to TNF $\alpha$  alone <sup>12</sup>.  
2 Alternative pathways might be of interest. For instance, the *IL33* gene is synergistically induced by  
3 IFN $\beta$ +TNF $\alpha$  in a STAT2 and IRF9-independent manner; scanning of the *IL33* promoter for  
4 transcription factor binding sites revealed AP-1, NF- $\kappa$ B and IRF7 consensus sites <sup>34</sup>. The potential  
5 role of AP-1 is also supported by the finding that the AP-1 transcription network module is enriched  
6 amongst IFN $\beta$ +TNF $\alpha$ -induced DEGs (**Figure 4E**). However, this module is not restricted to genes  
7 regulated independently of STAT2 and IRF9. It is also worth noting that two modules of IRF2-target  
8 genes were enriched, although again not specifically in the STAT2- and IRF-9 independent category  
9 (**Figure 4E**). Finally, increased colocalized recruitment of IRF1 and p65 to the promoter of a subset  
10 of genes induced by IFN $\alpha$  and TNF $\alpha$  in macrophages was observed <sup>35</sup>. However, while IRF1 was  
11 found synergistically induced by IFN $\beta$ +TNF $\alpha$  at early stages (**Figure 1**), we did not observe  
12 significant induction of IRF1 in the absence of STAT1 by RNASeq (**Supplemental Table S1**) or  
13 qRT-PCR (data not shown). Further studies will be needed to challenge the role of these various  
14 pathways in the synergistic induction of genes by IFN $\beta$ +TNF $\alpha$  independently of STAT2 and IRF9.

15  
16 Altogether our results demonstrate that in conditions with elevated levels of IFN $\beta$  and TNF $\alpha$ ,  
17 a broad antiviral and immunoregulatory delayed transcriptional program is elicited independently of  
18 STAT1. Our findings highlight the importance of diverse non-canonical STAT2 and/or IRF9  
19 pathways. Consistent with the growing literature, IFN $\beta$  and TNF $\alpha$  synergistic action is in part  
20 mediated by the concerted action of STAT2 and IRF9, most likely present in an alternative complex.  
21 Finally, our study reveals specific independent roles of STAT2 and IRF9 in the regulation of distinct  
22 sets of IFN $\beta$  and TNF $\alpha$ -induced genes.

23

## 1 MATERIAL AND METHODS

2

### 3 Cell culture and stimulation

4 A549 cells (American Type Culture Collection, ATCC) were grown in F-12 nutrient mixture (Ham)  
5 medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and 1% L-glutamine.  
6 The 2ftGH fibrosarcoma cell line and the derived STAT1-deficient U3A cell line, a generous gift  
7 from Dr. G. Stark, Cleveland, USA<sup>13</sup>, were grown in DMEM medium supplemented with 10% HI-  
8 FBS or HI-Fetal Clone III (HI-FCl) and 1% L-glutamine. U3A cells stably expressing STAT1 were  
9 generated by transfection of the STAT1 alpha flag pRc/CMV plasmid (Addgene plasmid #8691; a  
10 generous gift from Dr. J. Darnell, Rockefeller University, USA<sup>36,37</sup>) and selection with 800µg/ml  
11 Geneticin (G418). Monoclonal populations of U3A stably expressing STAT1 cells were isolated. A  
12 pool of two clones, referred to as U3A-STAT1, was used in the experiments to mitigate the clonal  
13 effects. U3A-STAT1 cells were maintained in culture in DMEM supplemented with 10% HI-FCl,  
14 1% Glu, and 200µg/ml G418. All cell lines were cultured without antibiotics. All media and  
15 supplements were from Gibco, with the exception of HI-FCl, which was from HyClone.  
16 Mycoplasma contamination was excluded by regular analysis using the MycoAlert Mycoplasma  
17 Detection Kit (Lonza). Cells were stimulated with IFNβ (1000 U/mL, PBL Assay Science), TNFα  
18 (10 ng/mL, R&D Systems) or IFNβ (1000 U/mL) +TNFα (10 ng/mL) for the indicated times.

19

### 20 siRNA Transfection

21 The sequences of non-targeting control (Ctrl) and STAT2- and IRF9-directed RNAi oligonucleotides  
22 (Dharmacon, USA) have previously been described in<sup>12</sup>. U3A cells at 30% confluency were  
23 transfected using the Oligofectamine transfection reagent (Life technologies). RNAi transfection was  
24 pursued for 48 h before stimulation.

1

## 2 **Immunoblot analysis**

3 Cells were lysed on ice using Nonidet P-40 lysis buffer as fully detailed in <sup>38</sup>. Whole-cell extracts  
4 (WCE) were quantified using the Bradford protein assay (Bio-Rad), resolved by SDS-PAGE and  
5 transferred to nitrocellulose membrane before analysis by immunoblot. Membranes were incubated  
6 with the following primary antibodies, anti-actin Cat #MAB1501 from Millipore, anti-IRF9 Cat  
7 #610285 from BD Transduction Laboratories, and anti-STAT1-P-Tyr701 Cat #9171, anti-STAT2-P-  
8 Tyr690 Cat #4441, anti-STAT1 Cat #9172, anti-STAT2 Cat #4594, all from Cell Signaling, before  
9 further incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (KPL or  
10 Jackson Immunoresearch Laboratories). Antibodies were diluted in PBS containing 0.5% Tween and  
11 either 5% nonfat dry milk or BSA. Immunoreactive bands were visualized by enhanced  
12 chemiluminescence (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer Life  
13 Sciences) using a LAS4000mini CCD camera apparatus (GE healthcare).

14

## 15 **RNA isolation and qRT-PCR analyses**

16 Total RNA was prepared using the RNAqueous-96 Isolation Kit (Ambion) following the  
17 manufacturer's instructions. Total RNA (1µg) was subjected to reverse transcription using the  
18 QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR were performed using either Fast  
19 start SYBR Green Kit (Roche) for *Mx1*, *IDO*, *APOBEC3G*, *CXCL10*, *NOD2*, *PKR*, *IRF1*, and *IFIT1*  
20 and *IL8* or TaqMan Gene Expression Assays (Applied Biosystems) for *DUOX2*, *IFI27*, *SERPINB2*,  
21 *IL33*, *CCL20*, *ISG20*, *OAS3*, *GRB14*, *CLEC5A*, *DGX58*, *ICAM1*, *IL1B*, *JUNB*, *MMP9*, *SERPINA1*  
22 and *HERC6*. Sequences of oligonucleotides and probes used in PCR reactions are described in  
23 **Supplemental Table S4**. Data collection was performed on a Rotor-Gene 3000 Real Time Thermal  
24 Cycler (Corbett Research). Gene inductions were normalized over S9 levels, measured using Fast

1 start SYBR Green Kit or TaqMan probe as necessary. Fold induction of genes was determined using  
2 the  $\Delta\Delta C_t$  method <sup>39</sup>. All qRT-PCR data are presented as the mean  $\pm$  SEM.

3

#### 4 **RNA-sequencing (RNASeq)**

5 Total RNA prepared as described above was quantified using a NanoDrop Spectrophotometer ND-  
6 1000 (NanoDrop Technologies, Inc.) and its integrity was assessed using a 2100 Bioanalyzer  
7 (Agilent Technologies). Libraries were generated from 250 ng of total RNA using the NEBNext  
8 poly(A) magnetic isolation module and the KAPA stranded RNA-Seq library preparation kit (Kapa  
9 Biosystems), as per the manufacturer's recommendations. TruSeq adapters and PCR primers were  
10 purchased from IDT. Libraries were quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit  
11 (Life Technologies) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa  
12 Biosystems). Average size fragment was determined using a LabChip GX (PerkinElmer) instrument.  
13 Massively parallel sequencing was carried out on an Illumina HiSeq 2500 sequencer. Read counts  
14 were obtained using HTSeq. Reads were trimmed from the 3' end to have a Phred score of at least 30.  
15 Illumina sequencing adapters were removed from the reads and all reads were required to have a  
16 length of at least 32bp. Trimming and clipping was performed using Trimmomatic <sup>40</sup>. The filtered  
17 reads were aligned to the Homo-sapiens assembly GRCh37 reference genome. Each readset was  
18 aligned using STAR <sup>41</sup> and merged using Picard (<http://broadinstitute.github.io/picard/>). For all  
19 samples, the sequencing resulted in more than 29 million clean reads (ranging from 29 to 44 million  
20 reads) after removing low quality reads and adaptors. The reads were mapped to the total of 63679  
21 gene biotypes including 22810 protein-coding genes. The non-specific filter for 1 count-per million  
22 reads (CPM) in at least three samples was applied to the reads and 14,254 genes passed this criterion.  
23 The entire set of RNAseq data has been submitted to the Gene Expression Omnibus (GEO) database  
24 (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE111195.

1

## 2 **Bioinformatics analysis**

3 Differential transcripts analysis. A reference-based transcript assembly was performed, which allows  
4 the detection of known and novel transcripts isoforms, using Cufflinks<sup>42</sup>, merged using Cuffmerge  
5 (cufflinks/AllSamples/merged.gtf) and used as a reference to estimate transcript abundance and  
6 perform differential analysis using Cuffdiff and Cuffnorm tool to generate a normalized data set that  
7 includes all the samples. FPKM values calculated by Cufflinks were used as input. The transcript  
8 quantification engine of Cufflinks, Cuffdiff, was used to calculate transcript expression levels in  
9 more than one condition and test them for significant differences. To identify a transcript as being  
10 differentially expressed, Cuffdiff tests the observed log-fold-change in its expression against the null  
11 hypothesis of no change (i.e. the true log-fold-change is zero). Because of measurement errors,  
12 technical variability, and cross-replicate biological variability might result in an observed log-fold-  
13 change that is non-zero, Cuffdiff assesses significance using a model of variability in the log-fold-  
14 change under the null hypothesis. This model is described in details in<sup>43</sup>. The differential gene  
15 expression analysis was performed using DESeq<sup>44</sup> and edgeR<sup>45</sup> within the R Bioconductor  
16 packages. Genes were considered differentially expressed between two group if they met the  
17 following requirement: fold change (FC) > ±1.5, p<0.05, FDR <0.05.

18 Enrichment of gene ontology (GO). GO enrichment analysis amongst differentially expressed genes  
19 (DEGs) was performed using Goseq<sup>46</sup> against the background of full human genome (hg19). GO-  
20 terms with adjusted p value < 0.05 were considered significantly enriched.

21 Clustering of DEGs. We categorized the DEGs according to their response upon silencing of  
22 siSTAT2 and siIRF9; categories are listed as A to I (Figure 2E). Then to determine relationship  
23 between these categories, we calculated the distance of centers of different categories. For each gene,  
24 we transformed siSTAT2 and siIRF9 fold changes (FC) to deviation from the mean FC of the

1 category the respective gene belongs:  $FC_{new} = FC_{old} - \varepsilon(FC_{category})$ . The parameter  $\varepsilon$  was estimated to  
2 give the perfect match between predefined categories (A to I) and clustering based on Euclidean  
3 distance. Results were plotted as a heatmap.

4 Modular transcription analysis. The *tmod* package in R <sup>47</sup> was used for modular transcription  
5 analysis. In brief, each transcriptional module is a set of genes, which shows coherent expression  
6 across many biological samples <sup>48, 49</sup>. Modular transcription analysis then calculates significant  
7 enrichment of a set of foreground genes, here DEGs, in pre-defined transcriptional module compared  
8 to a reference set. For transcriptional modules, we used a combined list of 606 distinct functional  
9 modules encompassing 12712 genes, defined by Chaussabel et al. <sup>14</sup> and Li et al. <sup>15</sup>, as the reference  
10 set in *tmod* package (**Supplemental Table S5**). The hypergeometric test devised in *tmodHGtest* was  
11 used to calculate enrichments and p-values employing Benjamini-Hochberg correction <sup>50</sup> for multiple  
12 sampling. All the statistical analyses and graphical presentations were in performed in R <sup>51</sup>.

13

#### 14 **Luciferase gene reporter assay**

15 U3A or U3A-STAT1 cells at 90% confluency were cotransfected with 100 ng of one of the  
16 following CXCL10 promoter containing firefly luciferase reporter plasmids (generously donated by  
17 Dr. David Proud, Calgary, <sup>52</sup>), CXCL10prom-972pb-pGL4 (full length -875/+97 promoter),  
18 CXCL10prom-376pb-pGL4 (truncated -279/+97 promoter), CXCL10prom972pb- $\Delta$ ISRE(3)-pGL4  
19 (full length promoter with ISRE(3) site mutated), CXCL10prom972pb- $\Delta$  $\kappa$ B(1)-pGL4 (full length  
20 promoter with NF $\kappa$ B(1) site mutated), CXCL10prom972pb- $\Delta$  $\kappa$ B(2)-pGL4 (full length promoter with  
21 NF $\kappa$ B(2) site mutated), CXCL10prom972pb- $\Delta$ AP-1-pGL4 (full length promoter with AP-1 site  
22 mutated) together with 50ng of pRL-null renilla-luciferase expressing plasmid (internal control).  
23 Transfection was performed using Lipofectamine 2000 (Life technologies) using a 1:2 DNA to  
24 lipofectamine ratio. At 8 h post-transfection, cells were stimulated for 16 h with either IFN $\beta$  or

1 IFN $\beta$ +TNF $\alpha$ . Firefly and renilla luciferase activities were quantified using the Dual-luciferase  
2 reporter assay system (Promega). Luciferase activities were calculated as the luciferase/renilla ratio  
3 and were expressed as fold over the non-stimulated condition.

4

#### 5 **Virus titration by plaque assay**

6 Quantification of VSV infectious virions was achieved through methylcellulose plaque forming unit  
7 assays. U3A and U3A-STAT1 cells were either left untreated or stimulated with IFN $\beta$  or  
8 IFN $\beta$ +TNF $\alpha$  for 30h. Cells were then infected with Vesicular Stomatitis Virus (VSV)-GFP (kindly  
9 provided by Dr. J. Bell, University of Ottawa, Canada) at an MOI of 5 for 1h in serum free medium  
10 (SFM). Cells were then washed twice with SFM and further cultured in DMEM medium containing  
11 2% HI-FCI. The supernatants were harvested at 12h post-infection and serial dilutions were used to  
12 infect confluent Vero cells (ATCC) for 1h in SFM. The medium was then replaced with 1%  
13 methylcellulose in DMEM containing 10% HI-FCI. Two days post-infection, GFP-positive plaques  
14 were detected using a Typhoon Trio apparatus and quantified using the Imagequant software  
15 (Molecular Dynamics).

16

#### 17 **Statistical analyses**

18 Statistical analyses of qRT-PCR and luciferase assay results were performed using the Prism 7  
19 software (GraphPad) using the tests indicated in the figure legends. Statistical significance was  
20 evaluated using the following *P* values: *P* < 0.05 (\*), *P* < 0.01 (\*\*), *P* < 0.001 (\*\*\*) or  
21 *P* < 0.0001 (\*\*\*\*). Differences with a *P*-value < 0.05 were considered significant. Statistical analysis  
22 of the RNASeq data is described in the Bioinformatics analysis section above.

23

24

## 1 **ACKNOWLEDGEMENTS**

2 We are very thankful to Dr. D. Proud (University of Calgary, Canada), Dr. G. Stark (Cleveland  
3 clinic, USA) and Dr. J. Bell (University of Ottawa, Canada) for sharing reagents used in this study.  
4 RNASeq analyses were performed at the McGill University and Génome Québec Innovation Centre.  
5 The present work was funded by grants from Natural Sciences and Engineering Research Council of  
6 Canada (NSERC RGPIN/355306-2012 and RGPIN/2018-04279) and by the Research Chair in  
7 signaling in virus infection and oncogenesis from the Université de Montréal to NG. AS  
8 acknowledges funds from the University of Montreal, NSERC and the Merck Foundation. SCO was  
9 the recipient of a MITACS Globalink studentship. NG was recipient of a Tier II Canada Research  
10 Chair.

11

## 12 **AUTHOR CONTRIBUTIONS**

13 MM and NG conceived and designed the experiments. MKM, AF, EC, MK, ANH, DK, SCO, and  
14 EM performed experiments. PD and AS performed bioinformatics analysis. MKM and NG analyzed  
15 the data. NG wrote the manuscript. All co-authors edited and approved the manuscript.

16

## 17 **CONFLICT OF INTEREST**

18 Authors declare no competing financial interests in relation to the work described in this study.

19

## 20 **REFERENCES**

21 1 McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. Type I interferons in infectious disease.  
22 *Nat Rev Immunol* 2015; **15**:87-103.



- 1 2 Tomasello E, Pollet E, Vu Manh TP, Uze G, Dalod M. Harnessing Mechanistic Knowledge on  
2 Beneficial Versus Deleterious IFN-I Effects to Design Innovative Immunotherapies Targeting  
3 Cytokine Activity to Specific Cell Types. *Frontiers in immunology* 2014; **5**:526.
- 4 3 Levy DE, Marie IJ, Durbin JE. Induction and function of type I and III interferon in response to  
5 viral infection. *Curr Opin Virol* 2011; **1**:476-486.
- 6 4 Fink K, Grandvaux N. STAT2 and IRF9: Beyond ISGF3. *JAKSTAT* 2013; **2**:e27521.
- 7 5 Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host  
8 defenses. *Annual review of immunology* 2014; **32**:513-545.
- 9 6 Au-Yeung N, Mandhana R, Horvath CM. Transcriptional regulation by STAT1 and STAT2 in the  
10 interferon JAK-STAT pathway. *JAKSTAT* 2013; **2**:e23931.
- 11 7 Majoros A, Platanitis E, Kernbauer-Holzl E, Rosebrock F, Muller M, Decker T. Canonical and  
12 Non-Canonical Aspects of JAK-STAT Signaling: Lessons from Interferons for Cytokine Responses.  
13 *Frontiers in immunology* 2017; **8**:29.
- 14 8 Cantaert T, Baeten D, Tak PP, van Baarsen LG. Type I IFN and TNFalpha cross-regulation in  
15 immune-mediated inflammatory disease: basic concepts and clinical relevance. *Arthritis Res Ther*  
16 2010; **12**:219.
- 17 9 Mestan J, Brockhaus M, Kirchner H, Jacobsen H. Antiviral activity of tumour necrosis factor.  
18 Synergism with interferons and induction of oligo-2',5'-adenylate synthetase. *J Gen Virol* 1988; **69** (  
19 **Pt 12**):3113-3120.
- 20 10 Barteel E, Mohamed MR, Lopez MC, Baker HV, McFadden G. The addition of tumor necrosis  
21 factor plus beta interferon induces a novel synergistic antiviral state against poxviruses in primary  
22 human fibroblasts. *J Virol* 2009; **83**:498-511.

- 1 11 Yarilina A, Park-Min KH, Antoniv T, Hu X, Ivashkiv LB. TNF activates an IRF1-dependent  
2 autocrine loop leading to sustained expression of chemokines and STAT1-dependent type I  
3 interferon-response genes. *Nat Immunol* 2008; **9**:378-387.
- 4 12 Fink K, Martin L, Mukawera E *et al.* IFNbeta/TNFalpha synergism induces a non-canonical  
5 STAT2/IRF9-dependent pathway triggering a novel DUOX2 NADPH oxidase-mediated airway  
6 antiviral response. *Cell Res* 2013; **23**:673-690.
- 7 13 McKendry R, John J, Flavell D, Muller M, Kerr IM, Stark GR. High-frequency mutagenesis of  
8 human cells and characterization of a mutant unresponsive to both alpha and gamma interferons.  
9 *Proc Natl Acad Sci U S A* 1991; **88**:11455-11459.
- 10 14 Chaussabel D, Quinn C, Shen J *et al.* A modular analysis framework for blood genomics studies:  
11 application to systemic lupus erythematosus. *Immunity* 2008; **29**:150-164.
- 12 15 Li S, Roupheal N, Duraisingham S *et al.* Molecular signatures of antibody responses derived from  
13 a systems biology study of five human vaccines. *Nat Immunol* 2014; **15**:195-204.
- 14 16 Bluysen HA, Levy DE. Stat2 is a transcriptional activator that requires sequence-specific  
15 contacts provided by stat1 and p48 for stable interaction with DNA. *J Biol Chem* 1997; **272**:4600-  
16 4605.
- 17 17 Martinez-Moczygemba M, Gutch MJ, French DL, Reich NC. Distinct STAT structure promotes  
18 interaction of STAT2 with the p48 subunit of the interferon-alpha-stimulated transcription factor  
19 ISGF3. *J Biol Chem* 1997; **272**:20070-20076.
- 20 18 Gupta S, Jiang M, Pernis AB. IFN-alpha activates Stat6 and leads to the formation of Stat2:Stat6  
21 complexes in B cells. *J Immunol* 1999; **163**:3834-3841.
- 22 19 Lou YJ, Pan XR, Jia PM *et al.* IRF-9/STAT2 [corrected] functional interaction drives retinoic  
23 acid-induced gene G expression independently of STAT1. *Cancer Res* 2009; **69**:3673-3680.

- 1 20 Abdul-Sater AA, Majoros A, Plumlee CR *et al.* Different STAT Transcription Complexes Drive  
2 Early and Delayed Responses to Type I IFNs. *J Immunol* 2015; **195**:210-216.
- 3 21 Perry ST, Buck MD, Lada SM, Schindler C, Shresta S. STAT2 mediates innate immunity to  
4 Dengue virus in the absence of STAT1 via the type I interferon receptor. *PLoS Pathog* 2011;  
5 **7**:e1001297.
- 6 22 Wienerroither S, Shukla P, Farlik M *et al.* Cooperative Transcriptional Activation of  
7 Antimicrobial Genes by STAT and NF-kappaB Pathways by Concerted Recruitment of the Mediator  
8 Complex. *Cell Rep* 2015; **12**:300-312.
- 9 23 Farlik M, Reutterer B, Schindler C *et al.* Nonconventional initiation complex assembly by STAT  
10 and NF-kappaB transcription factors regulates nitric oxide synthase expression. *Immunity* 2010;  
11 **33**:25-34.
- 12 24 Lau JF, Nusinzon I, Burakov D, Freedman LP, Horvath CM. Role of metazoan mediator proteins  
13 in interferon-responsive transcription. *Mol Cell Biol* 2003; **23**:620-628.
- 14 25 Zakharova N, Lymar ES, Yang E *et al.* Distinct transcriptional activation functions of  
15 STAT1alpha and STAT1beta on DNA and chromatin templates. *J Biol Chem* 2003; **278**:43067-  
16 43073.
- 17 26 Nan J, Wang Y, Yang J, Stark GR. IRF9 and unphosphorylated STAT2 cooperate with NF-  
18 kappaB to drive IL6 expression. *Proc Natl Acad Sci U S A* 2018; **115**:3906-3911.
- 19 27 Wan L, Lin CW, Lin YJ *et al.* Type I IFN induced IL1-Ra expression in hepatocytes is mediated  
20 by activating STAT6 through the formation of STAT2: STAT6 heterodimer. *J Cell Mol Med* 2008;  
21 **12**:876-888.
- 22 28 Ghislain JJ, Fish EN. Application of genomic DNA affinity chromatography identifies multiple  
23 interferon-alpha-regulated Stat2 complexes. *J Biol Chem* 1996; **271**:12408-12413.

- 1 29 Suprunenko T, Hofer MJ. The emerging role of interferon regulatory factor 9 in the antiviral host  
2 response and beyond. *Cytokine Growth Factor Rev* 2016; **29**:35-43.
- 3 30 Li W, Hofer MJ, Songkhunawej P *et al.* Type I interferon-regulated gene expression and signaling  
4 in murine mixed glial cells lacking signal transducers and activators of transcription 1 or 2 or  
5 interferon regulatory factor 9. *J Biol Chem* 2017; **292**:5845-5859.
- 6 31 Zhao X, Chu Q, Cui J, Huo R, Xu T. IRF9 as a negative regulator involved in TRIF-mediated NF-  
7 kappaB pathway in a teleost fish, *Miichthys miiuy*. *Mol Immunol* 2017; **85**:123-129.
- 8 32 Tian WL, Guo R, Wang F *et al.* IRF9 inhibits human acute myeloid leukemia through the SIRT1-  
9 p53 signaling pathway. *FEBS Lett* 2017.
- 10 33 Cheshire JL, Baldwin AS, Jr. Synergistic activation of NF-kappaB by tumor necrosis factor alpha  
11 and gamma interferon via enhanced I kappaB alpha degradation and de novo I kappaBbeta  
12 degradation. *Mol Cell Biol* 1997; **17**:6746-6754.
- 13 34 Sun L, Zhu Z, Cheng N, Yan Q, Ye RD. Serum amyloid A induces interleukin-33 expression  
14 through an IRF7-dependent pathway. *Eur J Immunol* 2014; **44**:2153-2164.
- 15 35 Park SH, Kang K, Giannopoulou E *et al.* Type I interferons and the cytokine TNF cooperatively  
16 reprogram the macrophage epigenome to promote inflammatory activation. *Nat Immunol* 2017;  
17 **18**:1104-1116.
- 18 36 Horvath CM, Wen Z, Darnell JE, Jr. A STAT protein domain that determines DNA sequence  
19 recognition suggests a novel DNA-binding domain. *Genes Dev* 1995; **9**:984-994.
- 20 37 Schindler C, Fu XY, Improta T, Aebersold R, Darnell JE, Jr. Proteins of transcription factor  
21 ISGF-3: one gene encodes the 91- and 84-kDa ISGF-3 proteins that are activated by interferon alpha.  
22 *Proc Natl Acad Sci U S A* 1992; **89**:7836-7839.
- 23 38 Robitaille AC, Mariani MK, Fortin A, Grandvaux N. A High Resolution Method to Monitor  
24 Phosphorylation-dependent Activation of IRF3. *J Vis Exp* 2016:e53723.

- 1 39 Dussault AA, Pouliot M. Rapid and simple comparison of messenger RNA levels using real-time  
2 PCR. *Biol Proced Online* 2006; **8**:1-10.
- 3 40 Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.  
4 *Bioinformatics* 2014; **30**:2114-2120.
- 5 41 Dobin A, Davis CA, Schlesinger F *et al.* STAR: ultrafast universal RNA-seq aligner.  
6 *Bioinformatics* 2013; **29**:15-21.
- 7 42 Roberts A, Pimentel H, Trapnell C, Pachter L. Identification of novel transcripts in annotated  
8 genomes using RNA-Seq. *Bioinformatics* 2011; **27**:2325-2329.
- 9 43 Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of  
10 gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 2013; **31**:46-53.
- 11 44 Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol* 2010;  
12 **11**:R106.
- 13 45 Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential  
14 expression analysis of digital gene expression data. *Bioinformatics* 2010; **26**:139-140.
- 15 46 Young MD, Wakefield MJ, Smyth GK, Oshlack A. Gene ontology analysis for RNA-seq:  
16 accounting for selection bias. *Genome Biol* 2010; **11**:R14.
- 17 47 Weiner J, Domaszewska T. tmod: an R package for general and multivariate enrichment analysis.  
18 *PeerJ Preprints* 2016;4:e2420v2421.
- 19 48 Bar-Joseph Z, Gerber GK, Lee TI *et al.* Computational discovery of gene modules and regulatory  
20 networks. *Nat Biotechnol* 2003; **21**:1337-1342.
- 21 49 Chaussabel D, Baldwin N. Democratizing systems immunology with modular transcriptional  
22 repertoire analyses. *Nat Rev Immunol* 2014; **14**:271-280.

- 1 50 Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful  
2 Approach to Multiple Testing *Journal of the Royal Statistical Society Series B (Methodological)*  
3 1995; **57**: 289-300
- 4 51 Ross I, Gentleman R. R: a language for data analysis and graphics. *Journal of computational and*  
5 *graphical statistics* 1996; **5.3**:299-314.
- 6 52 Zaheer RS, Koetzler R, Holden NS, Wiehler S, Proud D. Selective transcriptional down-  
7 regulation of human rhinovirus-induced production of CXCL10 from airway epithelial cells via the  
8 MEK1 pathway. *J Immunol* 2009; **182**:4854-4864.
- 9

## 1 **FIGURE LEGENDS**

2

### 3 **Figure 1: Expression of a panel of antiviral and immunoregulatory genes in response to IFN $\beta$ ,** 4 **TNF $\alpha$ and IFN $\beta$ +TNF $\alpha$ .**

5 A549 cells were stimulated with either TNF $\alpha$ , IFN $\beta$  or costimulated with IFN $\beta$ +TNF $\alpha$  for the  
6 indicated times. Quantification of mRNA was performed by qRT-PCR and expressed as fold  
7 expression after normalization to the S9 mRNA levels using the  $\Delta\Delta$ Ct method. Mean +/- SEM, n $\geq$ 3.  
8 Statistical comparison of TNF $\alpha$  vs. IFN $\beta$ +TNF $\alpha$  and IFN $\beta$  vs. IFN $\beta$ +TNF $\alpha$  was conducted using  
9 two-way ANOVA with Tukey's post-test.  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*) or  $P < 0.0001$  (\*\*\*\*).

10

### 11 **Figure 2: Experimental design used to study the STAT1-independent delayed transcriptional** 12 **program induced by the combination of IFN $\beta$ and TNF $\alpha$ .**

13 **A)** U3A (STAT1-deficient) and 2ftGH (parental STAT1-positive) cells were left untreated or  
14 stimulated with IFN $\beta$ +TNF $\alpha$  for the indicated times. WCE were analyzed by SDS-PAGE followed  
15 by immunoblot using anti STAT1-P-Tyr701, total STAT1, STAT2-P-Tyr690, total STAT2, IRF9 or  
16 actin antibodies. **B-E)** U3A cells were transfected with siCTRL, siSTAT2 or siIRF9 before being left  
17 untreated (NS) or stimulated with IFN $\beta$ +TNF $\alpha$  for 24h. **In B**, The schematic describes the workflow  
18 of sample preparation and analysis. **In C**, WCE were analyzed by SDS-PAGE followed by  
19 immunoblot using anti STAT2, IRF9 and actin antibodies. **In D**, Graph showing the correlation  
20 between fold-changes (FC) measured by RNASeq and qRT-PCR for 13 genes. Data from siCTRL  
21 NS vs siCTRL IFN $\beta$ +TNF $\alpha$ , siSTAT2 IFN $\beta$ +TNF $\alpha$  vs siCTRL IFN $\beta$ +TNF $\alpha$ , siIRF9 IFN $\beta$ +TNF $\alpha$   
22 vs siCTRL IFN $\beta$ +TNF $\alpha$  conditions were used. **In E**, Diagram describing the bioinformatics analysis  
23 strategy used to determine STAT1-independent differentially expressed genes (DEGs) and their  
24 regulation by STAT2 and IRF9.

1  
2 **Figure 3: Functional characterization of STAT1-independent IFN $\beta$ +TNF $\alpha$ -induced DEGs.**  
3 **A)** Volcano plots of the fold-change (FC) vs. adjusted *P*-value of IFN $\beta$ +TNF $\alpha$  (I+T) vs non-  
4 stimulated (NS) siCtrl conditions. **B)** Gene ontology (GO) enrichment analysis of the differentially  
5 upregulated genes in IFN $\beta$ +TNF $\alpha$  vs non-stimulated siCtrl conditions based on the Biological  
6 Processes and Molecular Function categories. **C)** Modular transcription analysis of upregulated  
7 DEGs. Eighteen enriched modules are shown. The full list of enriched modules is available  
8 **Supplemental Table S3.** **D)** U3A and STAT1-rescued U3A-STAT1 cells were stimulated with IFN $\beta$   
9 (I) or IFN $\beta$ +TNF $\alpha$  for 30h before infection with VSV at a MOI of 5 for 12h. The release of  
10 infectious viral particles was quantified by plaque forming unit (pfu) assay. The left graphs show dot-  
11 plots of all stimulations. Statistical comparisons were performed on the “before and after” plots  
12 (displayed on the right of dot-plot graphs) using ratio paired t-tests. Top panel shows the immunoblot  
13 in 2ftGH, U3A-STAT1 and U3A cells using anti-STAT1 and anti-tubulin (loading control)  
14 antibodies.

15  
16 **Figure 4: Clustering of IFN $\beta$ +TNF $\alpha$ -induced DEGs according to their regulation by STAT2**  
17 **and IRF9.**

18 **A)** Volcano plots of the fold-change vs. adjusted *p*-value of siSTAT2 IFN $\beta$ +TNF $\alpha$  vs. siCTRL  
19 IFN $\beta$ +TNF $\alpha$  (I+T) conditions. **B)** Volcano plots of the fold-change vs. adjusted *p*-value of siIRF9  
20 IFN $\beta$ +TNF $\alpha$  vs. siCTRL IFN $\beta$ +TNF $\alpha$  conditions. **C)** Hierarchical clustering of the categories of  
21 DEG responses according to their regulation by STAT2 and IRF9. Euclidean distance metric is used  
22 for the construction of distance matrix and the categories are used *a priori* input into clustering  
23 algorithm as detailed in Materials and Methods. **D)** Validation of DEGs regulation profile by qRT-  
24 PCR. U3A cells were transfected with siCTRL (C), siSTAT2 (S2) or siIRF9 (F9). Cells were further



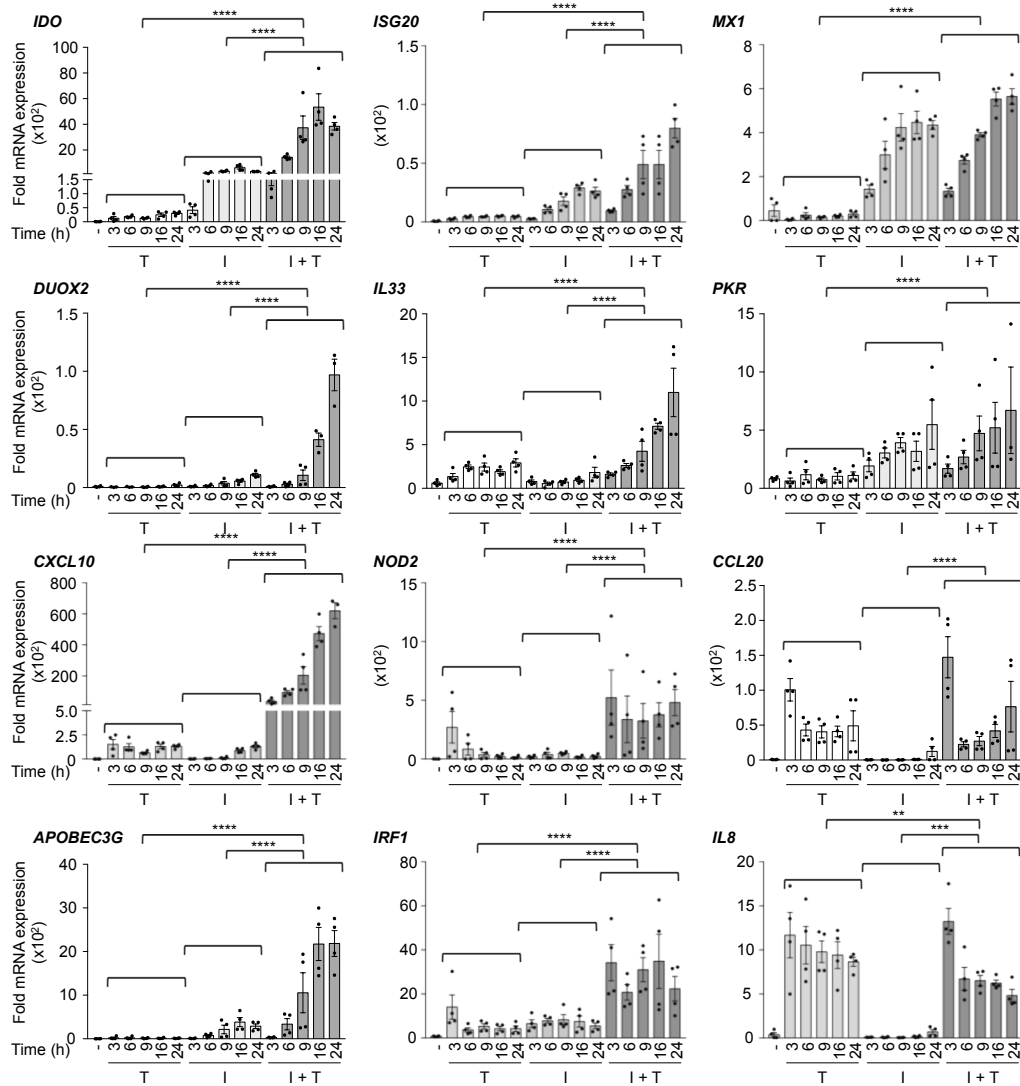
1 left untreated or stimulated with IFN $\beta$ +TNF $\alpha$  for 24h. Quantification of the mRNA corresponding to  
2 the indicated genes was performed by qRT-PCR and expressed as relative expression ( $\Delta\Delta C_t$ ) after  
3 normalization to the S9 mRNA levels. Mean +/- SEM, n $\geq$ 5. Statistical comparison of siSTAT2  
4 IFN $\beta$ +TNF $\alpha$  vs siCTRL IFN $\beta$ +TNF $\alpha$  and siIRF9 IFN $\beta$ +TNF $\alpha$  vs siCTRL IFN $\beta$ +TNF $\alpha$  conditions  
5 was conducted using one-way ANOVA with Dunett post-test.  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$   
6 (\*\*\*) or  $P < 0.0001$  (\*\*\*\*). **E)** Diagram showing enriched transcription modules in each gene  
7 category.

8

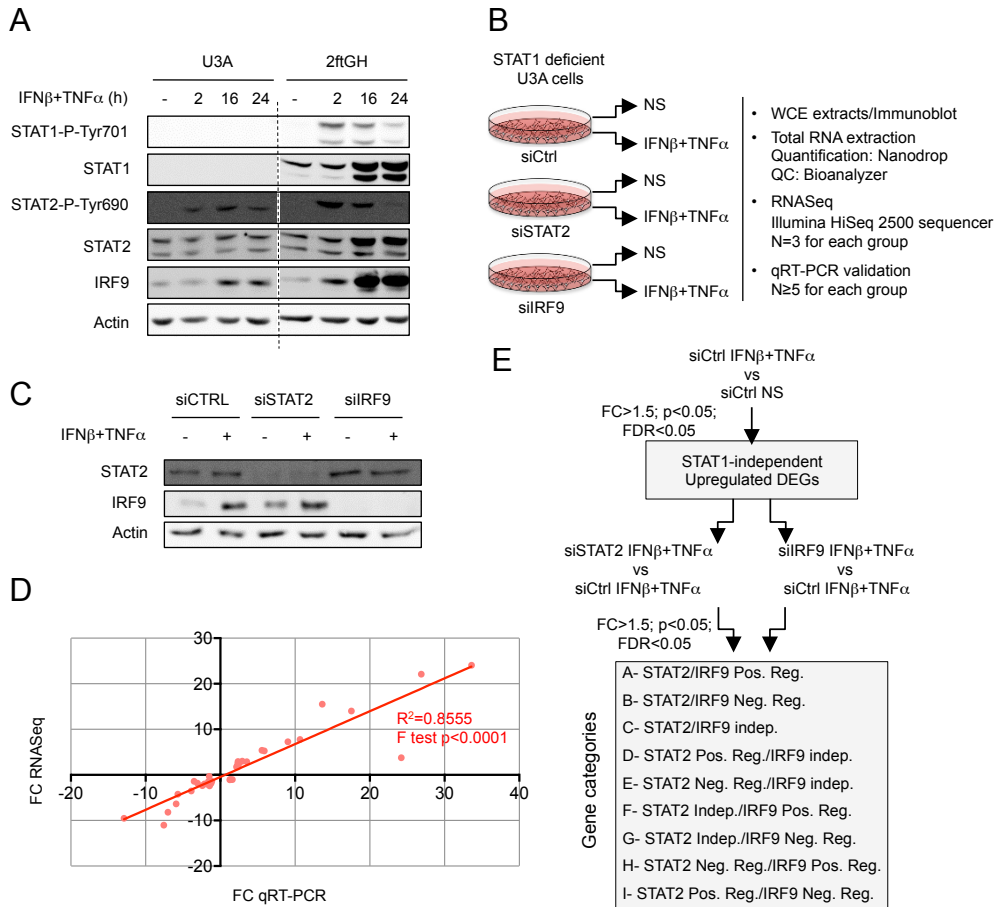
9 **Figure 5: Analysis of the *CXCL10* promoter regulation in response to IFN $\beta$ +TNF $\alpha$  vs IFN $\beta$ .**

10 **A)** Schematic representation of the *CXCL10* promoter (*CXCL10*prom) luciferase constructs used in  
11 this study indicating the main transcription factors consensus binding sites. **B)** U3A and U3A-STAT1  
12 cells were transfected with the indicated *CXCL10*prom-luciferase reporter constructs and either left  
13 untreated or stimulated with IFN $\beta$  or IFN $\beta$ +TNF $\alpha$ . Relative luciferase activities were measured at  
14 16h post-stimulation and expressed as fold over the corresponding unstimulated condition. Mean +/-  
15 SEM, n=6. Statistical analyses were performed using an unpaired t-test comparing each promoter to  
16 the *CXCL10*prom-972bp construct.  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*)).

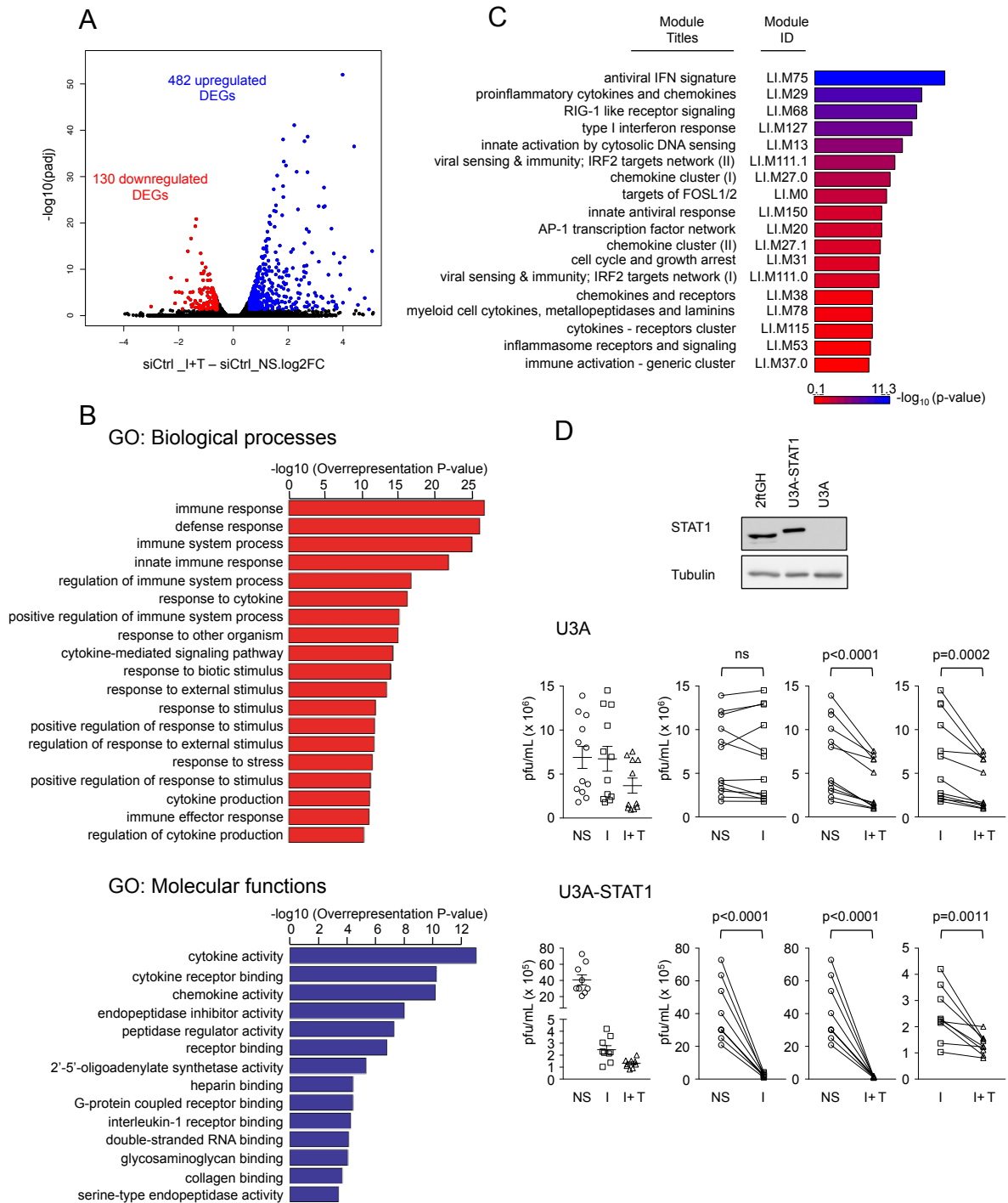
Mariani et al., Figure 1



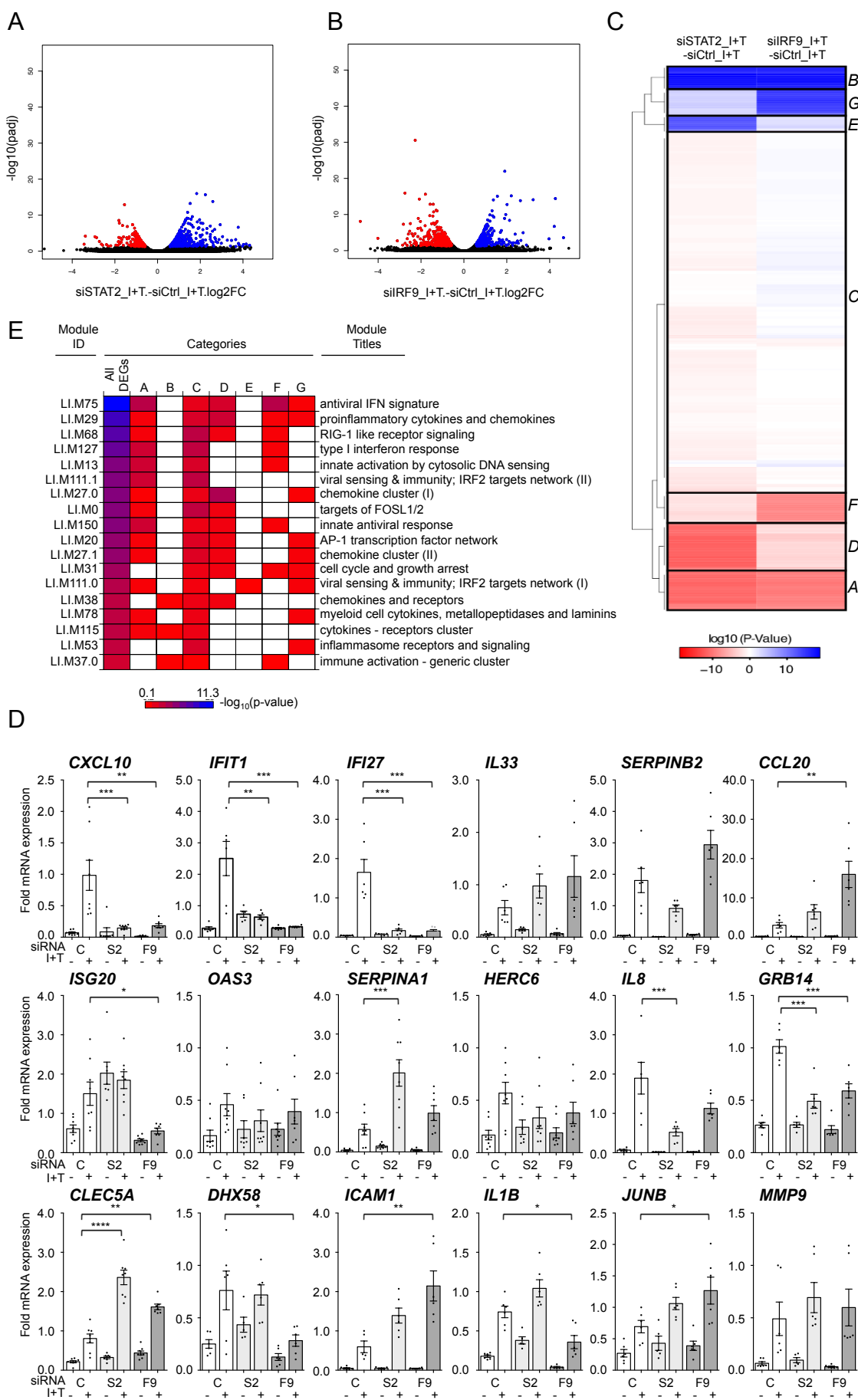
Mariani et al., Figure 2



Mariani et al., Figure 3

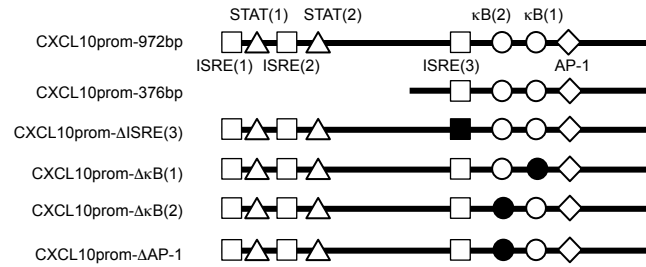


Mariani et al., Figure 4



Mariani et al., Figure 5

A



B

