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Asymmetric action of STAT transcription factors drive transcriptional outputs and cytokine specificity

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SUMMARY

Interleukin-6 (IL-6) and IL-27 signal through a shared receptor subunit and employ the same downstream STAT transcription proteins, but yet are ascribed unique and overlapping functions. To evaluate the specificity and redundancy for these cytokines, we quantified their global

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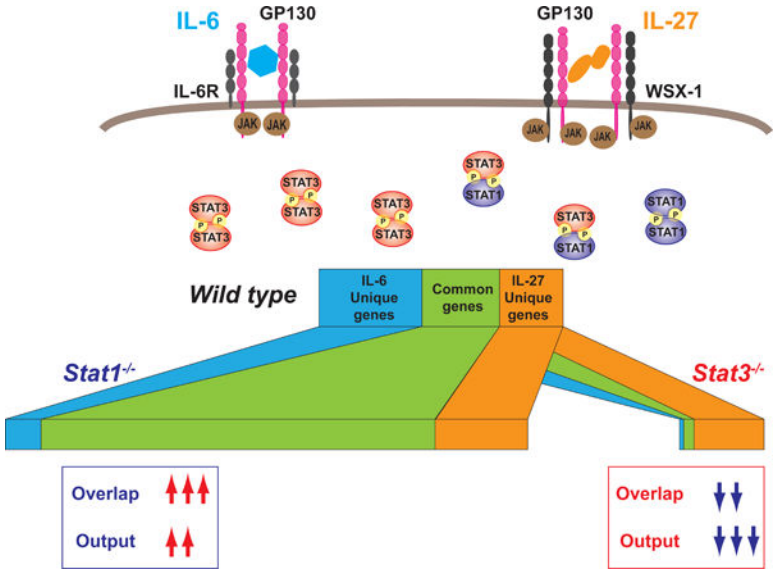
transcriptomic changes and determined the relative contributions of STAT1 and STAT3 using genetic models and chromatin immunoprecipitation-sequencing (ChIP-seq) approaches. We found an extensive overlap of the transcriptomes induced by IL-6 and IL-27 and few examples in which the cytokines acted in opposition. Using STAT-deficient cells and T cells from patients with gain-of-function *STAT1* mutations, we demonstrated that STAT3 is responsible for the overall transcriptional output driven by both cytokines, whereas STAT1 is the principal driver of specificity. STAT1 cannot compensate in the absence of STAT3 and, in fact, much of STAT1 binding to chromatin is STAT3 dependent. Thus, STAT1 shapes the specific cytokine signature superimposed upon STAT3's action.

Graphical Abstract



INTRODUCTION

Cytokines are a diverse group of factors that regulate hematopoiesis and host defense, as well as homeostasis, metabolism and growth. Despite many distinct activities, widespread functional redundancy is an unforeseen aspect of cytokine biology, and a critical departure from the "one cytokine one function" theme that dominated in the early cytokine research (Paul, 1989). However, the advent of cytokine- and cytokine receptor deficient mice has emphasized non-redundant functionalities, as manifested by distinct *in vivo* phenotypes. Reconciling specificity in the context of redundancy and deciphering the molecular underpinning for both functional aspects has been a longstanding conundrum in cytokine biology.

Interleukin-27 (IL-27) was first recognized to induce interferon (IFN)-γ, T-bet and IL-12Rβ2 (Hibbert et al., 2003; Pflanz et al., 2002). However, mice lacking IL-27 responsiveness clearly demonstrate that the essential, non-redundant function of IL-27 is constraining immune responses (Villarino et al., 2003). The mechanisms underlying IL-27's immunosuppressive aspects include inhibition of IL-17, the transcription factors Rorγt,

Rorα, GATA-3, and the growth and differentiation factor GM-CSF (Diveu et al., 2009; Lucas et al., 2003; Stumhofer et al., 2006; Young et al., 2012). IL-27 also induces PD-L1 (Hirahara et al., 2012), IL-10 (Awasthi et al., 2007; Stumhofer et al., 2007) and promotes the differentiation of T regulatory 1 (Tr1) cells (Apetoh et al., 2010; Awasthi et al., 2007). *IL27* polymorphisms with chronic obstructive pulmonary disease, inflammatory bowel disease and diabetes (Barrett et al., 2009; Huang et al., 2008; Imielinski et al., 2009) also support immunoregulatory roles.

IL-27 binds to a receptor composed of a ligand-specific subunit, termed WSX-1, and glycoprotein 130 (gp130) (Pflanz et al., 2004). Gp130 is a shared signaling subunit also used by IL-6 (Taga et al., 1989). This is notable since IL-6 and IL-27 have been characterized as have opposing functions: IL-6 inhibits Th1 cell differentiation, enhances Th2 cell differentiation and promotes Th17 cell differentiation, whereas IL-27 promotes Th1 cell differentiation and inhibits both Th2 and Th17 cell differentiation (Bettelli et al., 2006; Diehl et al., 2000; Rincon et al., 1997). Like other members of the hematopoietin family of receptors, IL-27 and IL-6 exert their effect through Janus kinases and signal transducer and activator of transcription (STATs), mainly via a combination of STAT3 and STAT1 (Lucas et al., 2003; Takeda et al., 2003; Villarino et al., 2003). The pro-inflammatory actions of IL-6 have been attributed to STAT3, whereas the ability to activate STAT1 has been argued to explain the “Th1” cell features of IL-27, as well as its ability to inhibit Th17 cells (Villarino et al., 2010). However, immunosuppressive functions of IL-27 have also been attributed to STAT3 (Pot et al., 2011). The ability of both cytokines to activate both STATs seems paradoxical given their distinct functions and it remains unclear how STAT3, for example, can support both the immunosuppressive actions of IL-27 and the proinflammatory effects of IL-6.

Because of the distinct functions of IL-6 and IL-27 in the context of shared signaling mechanisms, we investigated the questions of cytokine redundancy and specificity in this system. Using genome-wide approaches that permit comprehensive, quantitative analyses, we found that IL-6 and IL-27 regulated many of the same genes, but despite the commonalities in signal transduction, also had discrete transcriptomic profiles.

Mechanistically, we found that STAT3 controlled the overall magnitude of transcriptional outputs driven by IL-6 and IL-27 while STAT1 diversified their transcriptional responses. STAT1 did not compensate for STAT3 on a genomic level and most STAT1 binding to chromatin was dependent on the availability of STAT3. Rather, STAT1 drove the distinct effects of these cytokines, as evidenced by T cells from STAT1 deficient mice and also from patients with *STAT1* gain-of-function mutations. Thus, STAT3 is fundamental for both IL-6 and IL-27 response whereas STAT1 serves to shape unique cytokine signatures.

RESULTS

IL-6- and IL-27- transcriptomes exhibit considerable overlap

To explore the unique and redundant functions of IL-6 and IL-27, we sought to define global transcriptional changes using activated $CD4^+$ helper T cells as a model system. First we confirmed that activated T cells express receptors for both cytokines (Figure S1A), and

then measured gene expression by RNA-seq in response to exogenous IL-6 or IL-27 using activated untreated (i.e. no added cytokines) cells as a control.

We found that IL-6 induced 481 genes and repressed 401 genes (>2 fold change) whereas IL-27 induced 331 genes and repressed 359 genes (Figure 1A and S1B). More than half (n=503) of IL-6-regulated genes (n=882) were unique to this cytokine (Figure 1A) and 311 of 690 IL-27-regulated genes were selectively regulated by IL-27 (Figure 1A). Thus, the proportion of genes commonly regulated by IL-6 and IL-27 was roughly equivalent to the uniquely regulated genes.

Considering the opposing biological functions of these two cytokines, we expected that a substantial subset of genes would be oppositely regulated. However only a very small proportion of genes (22 genes) were oppositely regulated by IL-6 and IL-27 (Figure 1A asterisk and 1B). Nonetheless their identity was noteworthy; they included key immunoregulatory factors, such as *Ifng*, *Ccl5,* and *Rorc* (Figure 1B).

The opposing regulation of *Ifng* by IL-27 and IL-6 was notable in view of the reported effects of IL-27 and IL-6 as “Th1” and “Th2” cell-associated cytokines respectively (Rincon et al., 1997). Therefore, we compared the transcriptional signature of IL-6 and IL-27 to gene expression profiles of canonical helper T cell subsets (Figure 1C to 1H). Both IL-6 and IL-27 contributed similarly to Th1 versus Th2 cell-associated transcriptional signatures (Figure 1C and 1D). In fact, closer examination of individual “Th1 or Th2” cell-associated genes revealed variegated patterns (Figure 1E).

The immunoregulatory actions of IL-6 and IL-27 also relate to their effects on promoting or inhibiting immune responses (Hunter and Kastelein, 2012; Korn et al., 2009). Therefore, we compared the IL-6- and IL-27-regulated genes to programs associated with Th17 or inducible T regulatory (iTreg) cells. There was little global distinction between IL-6 and IL-27 with respect to the spectrum of gene expression characteristic of Th17 or iTreg cells (Figure 1F and 1G). IL-6 and IL-27 elicited similar as well as opposing effects on key genes (Figure 1H). Taken together, our comparative transcriptomic analyses revealed a degree of redundancy in gene sets regulated by IL-6 and IL-27, but also identified several key genes that did not conform to the expected general trend of coordinated regulation elicited by these two cytokines. Thus, the actions of IL-6 and IL-27 on T cells are *sui generis*; trying to categorize them as “Th1 cell-like”, Th2 cell-like” and pro- or anti-inflammatory is overly simplistic and belies important aspects of their function.

IL-27 and IL-6 dynamically activate STAT1 and STAT3

IL-27 and IL-6 employ multiple STATs, but are most associated with STAT1 and STAT3 (Lucas et al., 2003; Takeda et al., 1998; Villarino et al., 2003). However, it is known that expression of STAT proteins is dynamic (Gil et al., 2012). Therefore, we measured expression of STAT1 and STAT3 and found that both were induced in anti-CD3- and anti-CD28-activated $CD4^+$ T cells, but the inclusion of IL-6 or IL-27 in cultures did not alter expressions further (Figure 2A to 2C). In terms of STAT activity, IL-27 induced phosphorylation of STAT1 and STAT3 with the former being more prominent (Figure S2A to S2C). IL-6 also induced robust phosphorylation of both. Under the conditions employed,

IL-27 and IL-6 did not enhance phosphorylation of other STATs beyond what was seen in untreated cells (Figure S2B and S2C).

Next, we evaluated the dynamics of STAT activation by flow cytometry and found that IL-27 and IL-6 initially activated STAT1 to comparable amounts. Over the course of 72 hours, IL-6-mediated STAT1 activation declined but IL-27-dependent STAT1 activation increased (Figure 2D left panel). IL-6 initially activated STAT3 more than IL-27 but at later time points, IL-27 activated STAT3 more than IL-6 (Figure 2D right panel). In the absence of STAT3, STAT1 activation was increased but without STAT1, STAT3 activation was unchanged.

We also measured STAT activation by electrophoretic mobility shift assay (EMSA) (Figure 2E and 2F). At 24 hours post-stimulation, IL-27 induced roughly equivalent amounts of STAT1 and STAT3 homodimers, and STAT1-STAT3 heterodimers. IL-6 induced more STAT3 homodimers and STAT1-STAT3 heterodimers but little STAT1 homodimers. These data are consistent with the notion that IL-27 and IL-6 each employ STAT1 and STAT3, but and also suggest that STAT binding induced by the two cytokines is highly dynamic in terms of duration, intensity, and dimer composition.

We also tested the potential contribution of other signaling pathways that included AKT, Erk1, Erk2, IRS-1, GSK-3A and GSK-3B (Figure S2D), but found no significant differences between IL-6 and IL-27 in our experimental system.

STAT3 regulates the transcriptional outputs of IL-6 and IL-27

To determine the functional contribution of STAT1 and STAT3 for IL-6 and IL-27, we assessed the transcriptomic impact of STAT-deficiency. For genes selectively controlled by IL-6, regulation was profoundly affected by the absence of STAT3 (> 90%); a much smaller proportion (20.3%) was STAT1-dependent (Figure 3A, 3B, 3H and S3A). For genes commonly regulated by IL-6 and IL-27, the influence of STAT3 was still dominant (74.9%) over that of STAT1 (20.6%) (Figure 3C, 3D, and 3H). For genes selectively regulated by IL-27, the contribution of STAT1 was more prominent (65.0%), although the influence of STAT3 was still profound (76.8%) (Figure 3E to 3H). Only 42 genes (13.5%) were strictly STAT1-dependent and 79 genes (25.4%) were completely STAT3-dependent (Figure 3G). These data reveal a broad contribution of STAT3 to the overall transcriptional output of both IL-6 and IL-27, indicating that STAT1 cannot compensate in the absence of STAT3. Representative examples are shown in Figure S3B.

STAT1 drives the specificity of IL-27 versus IL-6

Next, we focused on the role of STAT1 on the transcription programs. We found that most IL-6 unique and common genes were preserved in the absence of STAT1 (Figure 3A and 3C). In the absence of STAT1, however, the genes regulated by IL-6 or IL-27 now largely overlapped (726 genes of 811 genes; 89.5%), indicating a loss of cytokine specificity (Figure 4A right panel). Furthermore, consistent with a previous report (Gil et al., 2001), the absence of STAT1 resulted in a gain of function phenotype such that IL-6 and IL-27 induced 1741 *de novo* genes that were not normally seen in wild type (Figure 4B). Overall, 2552 genes were regulated by IL-6 and IL-27 in STAT1 deleted cells, and cytokine response of

these genes was highly correlated between IL-6 and IL-27 (r=0.9823, Pearson's coefficient) compared with that of wild type (r=0.6482) (Figure 4C). This was not the case with cells lacking STAT3 in which very few *de novo* genes were observed (Figure S4A) and cytokine specificity was maintained (Figure S4B).

Asymmetric roles of STAT3 and STAT1 in transcriptomic responses

RNA-seq analysis of STAT-deficient cells argued that STAT1 and STAT3 fundamentally differ in their ability to affect transcriptional programs. To further explore this issue, we analyzed three categories of genes in terms of cytokine response (IL-6 unique, common, IL-27 unique) and assessed the fate of individual genes in STAT-deficient cells (Figure 5A and 5B). The major consequence of STAT1 deficiency was an expansion of genes co-regulated by IL-6 and IL-27 (Figure 5A, right side), whereas STAT3 deficiency was associated with the loss of overall cytokine response in all categories (Figure 5B, right side). The remaining genes were mainly IL-27-specific because they retained their original specificity or acquired an aberrant, new "specific" response (Figure 5B).

Therefore, when all IL-6 and IL-27 responsive genes (1193 genes in total) were evaluated in detail, we observed stark contrast between STAT3 and STAT1 regarding their global impact on cytokine specificity versus transcriptional output. A similar result was obtained when the expression of all genes was assessed (Figure 5C). Clear separation of clusters was evident corresponding to the three genotypes used (Wild type, *Stat1*$^{-/-}$, *Stat3*$^{-/-}$). In Wild type cells, the transcriptomes of IL-27- and IL-6-treated cells were distinct from each other and untreated cells. The separation between IL-6- and IL-27-stimulated cells was lost in the *Stat1*$^{-/-}$ cells, but there was still separation from untreated cells. In the *Stat3*$^{-/-}$ cells, all distinction was lost, as evidenced by the convergence of the untreated, IL-6 and IL-27 groups within this cluster.

We next evaluated the impact of STAT deficiency on transcriptomic changes over time (Figure 5D). At very early time points after stimulation (6 hours), loss of STAT1 had relatively little effect on IL-6-unique genes, with a greater effect on IL-27-unique genes. Over time, the transcriptomes induced by IL-6 and IL-27 expanded in wild type and the loss of transcriptome output associated with STAT3 deficiency became more profound. Similarly, the expansion of common genes was more apparent in STAT1-deficient cells at later time points. Presumably, this represents the integration of direct and indirect effects of autocrine cytokines, but regardless, the distinctive functions of STAT1 and STAT3 are very evident.

STAT1 and STAT3 dynamically bind to chromatin in an asymmetric fashion

We next assessed how genome-wide STAT3 and STAT1 distribution relates to the transcriptional activities of IL-27 and IL-6. Using STAT ChIP-seq, we found that some STAT1 and STAT3 peaks were stable, but cytokines markedly induced the genomic occupancy of both STATs (Figure 6A and 6B). A fraction of peaks were lost after cytokine stimulation. These data emphasize the contribution of STAT1 and STAT3 to the responses of both cytokines (Figure 6B). Canonical STAT binding motif (i.e. the GAS motif) was more prominent in STAT3 peaks than STAT1 peaks, particularly within peaks induced by both IL-6 and IL-27 (Figure 6C).

Next, we assessed STAT binding around differentially regulated genes (Figure 6D, S5A, and Table S1). Comparable binding of STAT3 and STAT1 was observed near genes that were regulated by IL-6. However, STAT3 binding was reduced compared to STAT1 near genes that were negatively regulated by IL-6, possibly indicating repressive effect of STAT1 for this category of genes.

A major issue in STAT biology is the extent to which different STATs can compensate for one another. This is especially the case for STAT1 and STAT3 (Costa-Pereira et al., 2002; Qing and Stark, 2004; Regis et al., 2008; Schiavone et al., 2011). Therefore we measured the binding of STAT1 and STAT3 in the presence and absence of the opposing STAT. Consistent with the asymmetric impact of each STAT deletion on transcriptome, we found that genome-wide STAT3 binding increased in *Stat1*$^{-/-}$ cells, whereas, STAT1 binding was significantly diminished in *Stat3*$^{-/-}$ cells (Figure S5B). This latter effect was quite evident for both IL-6 unique and commonly regulated genes (Figure 6D left and middle panels). However, for IL-27 uniquely regulated genes, STAT1 binding was relatively preserved in *Stat3*$^{-/-}$ cells, indicating a contribution of STAT1 independent of STAT3 (Figure 6D right panel). For genes whose cytokine specificity was altered in the absence of one STAT, STAT3 binding showed compensatory increase in the absence of STAT1 while STAT1 binding showed various degree of loss in the absence of STAT3 (Figure S5A). The functional consequences of this effect were confirmed for IFN-γ and Rorγt at protein amounts. (Figure S5C and S5D). Overall STAT1 preferential binding and influence become more evident when a defined subgroup of genes was evaluated, namely IL-6 down-regulated or IL-27 uniquely regulated genes.

Given that STAT1 and STAT3 can form homo- and heterodimers, we sought to investigate the possible generation of such complexes by examining regions in which binding of two STATs coincide or not. To quantify the likelihood of homo- and hetero-dimer binding, we calculated the ratio of STAT1 and STAT3 signals at peak locations and segregated them into three bins according to the three possible STAT1 and STAT3 configurations. Peak locations were also linked to the nearest genes as possible target of regulation (Figure 6E). In total, we observed 1.7 fold more STAT3 peaks than STAT1 peaks genome-wide. Of these, 33% of STAT3 peaks overlap with 56% of STAT1 peaks (Figure 6E: Venn diagram). Across all cytokine-inducible genes, the fraction of potential STAT3-STAT3 homodimers predominated. By contrast, there were relatively few regions bound only by STAT1. Notably, these were most often found that were in proximity to IL-6 down-regulated genes. These findings support the idea that STAT3 dominantly regulates cytokine responsive genes by working both singly (homodimers) and cooperatively (heterodimers).

Human *STAT1* gain of function mutation leads to aberrant transcriptome profiles

Gain-of-function (GOF) *STAT1* mutations are associated with a rare primary immunodeficiency with fungal infections (van de Veerdonk et al., 2011). We used T cells from these patients as a model to evaluate the impact of mutant STAT1 on transcriptomes regulated by IL-27 and IL-6 (Table S2). Compared to controls, T cells from *STAT1* GOF patients all exhibited a marked expansion of cytokine inducible genes (Figure 7A). There was a trend in the expansion of IL-6 regulated genes and a significant increase in IL-27-

regulated genes in the patient group (Figure 7B). Accordingly, the correlation coefficient between IL-6 and IL-27 responsive genes was significantly reduced in T cells from patients with *STAT1* GOF mutations (Figure 7C). Many of the genes affected by *STAT1* GOF mutations were immunologically relevant, and thus likely contribute to the pathologies associated with this genetic mutation (Table S3 and S4). Taken together, these findings are consistent with the idea that STAT1 contributes to transcriptomic diversity in response to cytokines.

DISCUSSION

Cytokines comprise a diverse group of factors such as interleukins, interferons and colony stimulating factors, as well as classic hormones including growth hormone, erythropoietin and prolactin. Understanding the redundant and specific actions of cytokines has been a major challenge in the field. In this study, we focused on two cytokines, IL-6 and IL-27, which share a receptor subunit (GP130) and two downstream signaling molecules (STAT1 and STAT3) but have distinct biological actions. Using genomic approaches, we generated a comprehensive view of the action of these highly related cytokines and examined the role of STATs in forming the distinctive transcriptomes.

A major goal of our study was to understand molecular underpinning to define specificity and redundancy of cytokine response through the action of STAT proteins. STAT family molecules are evolutionally conserved across a diverse range of organisms and expanded during evolution along with robust expansion of upstream cytokine receptors after the emergence of adaptive immunity (Gorissen et al., 2011; Liongue et al., 2012). There are 7 mammalian STATs and circumstances in which a given cytokine can be strongly linked to a particular STAT; such is the case of IL-12 and STAT4 (Kaplan et al., 1996; Levy and Darnell, 2002; O'Shea et al., 2002). From this perspective, IL-6 has often been equated with STAT3 and IL-27 with STAT1 (Takeda et al., 2003; Takeda et al., 1998). However, there are more than 60 cytokines that signal via 7 STATs and the mismatch in numbers means that many cytokines activate the same STATs and the action of cytokines likely represents the combinatorial engagement of multiple STATs.

The notion that access to different STATs influences the outcome of cytokine signaling has been articulated previously. One view is that in the absence of its preferred STAT, a given cytokine will acquire an alternative program through other STATs (Costa-Pereira et al., 2002). Indeed, IL-6 can regulate selected STAT1-inducible genes in STAT3-deficient cells (Costa-Pereira et al., 2002). Based on these findings, it might be predicted that in the absence of STAT3, IL-6 would approximate the action of IL-27 through activation of STAT1. Although this might be true for a subset of genes, our data clearly show that it is not the case on the genome-wide scale. Rather, our data indicate that STAT3 is the engine that drives transcriptional outputs of both cytokines, and it cannot be substituted by STAT1. Instead the major function of STAT1 is to provide the specificity that distinguishes IL-27 and IL-6. Our findings clearly demonstrate asymmetric roles for STAT3 and STAT1 and therefore differ substantially from previous views in which STAT1 and STAT3 were considered to have compensatory or overlapping roles (Costa-Pereira et al., 2002; Qing and Stark, 2004; Regis et al., 2008; Schiavone et al., 2011).

Our findings on the genome-wide distribution of STATs are consistent with this asymmetric view. STAT3 occupies wider region of chromatin than STAT1, often as homodimers and also likely in cooperation with STAT1 as heterodimers. An unanticipated finding was the collapse of global STAT1 binding in the absence of STAT3. Interestingly, the few remaining STAT1 bound regions present in the absence of STAT3 occur in proximity to genes selectively regulated by IL-27 or genes down regulated by IL-6, thereby contributes to the specificity of IL-6 and IL-27 action. Because ChIP-seq relies on the efficiency of any given antibody, we cannot make definitive conclusions about the absolute numbers of genomic STAT3 versus STAT1 binding sites. However the dominance of STAT3 peaks over STAT1 peaks is entirely consistent with the transcriptomic impact of loss of STAT3 versus STAT1.

The distinctive modes of action of STAT3 and STAT1 may reflect evolution of cytokine pathways. Gp130 is considered as a primordial receptor subunit, with homologues present in species such as tunicates and insects. In contrast, IL-27 appears to have arisen more recently during the evolution in Euteleostomes, along with other components of the vertebrate immune system. It is tempting to speculate that newer cytokines like IL-27 commandeered existing scaffolds (gp130) and achieve selectivity by engaging an additional receptor, in this case IL-27RA (WSX-1), which permits increased access to STAT1. This would explain why the absence of STAT3 cripples both IL-6 and IL-27 signaling and why STAT1 is the major driver of specificity. Previous reports support the notion that cytokine receptors can access more than one STAT protein and that this can be means through which output can be dynamically controlled. For instance, the priming of macrophages with IFN-γ resulted in increased STAT1 expression, thereby altering the cytokine response (Hu et al., 2002; Qiao et al., 2013). Likewise, the dynamic balance between STAT4 and STAT1 is crucial in modulating the course of type I interferon responses during viral infection in T and NK cells (Gil et al., 2012; Miyagi et al., 2007). Our findings using cells from patients with *STAT1* GOF mutations are in line with these findings by showing that altering the balance between STAT3 and STAT1 leads to global alteration in cytokine response.

Obviously, there are other factors that contribute to the specific of cytokine action. One is the duration of STAT signaling, and factors attenuate STAT activation such as suppressor of cytokine signaling (SOCS) proteins (Whitmarsh et al., 2011; Yasukawa et al., 2003). We observed that IL-27 preferentially induced SOCS1 whereas IL-6 preferentially induced SOCS3 (data not shown). Deletion of STAT1 abrogated IL-27 preferential SOCS1 induction while SOCS3 induction was heavily STAT3 dependent (data not shown). Therefore, regulation of SOCS expression is undoubtedly additional factors that may contribute to the distinctive cytokine signatures.

The other important factor to consider with respect to duration of signaling is the contribution of cytokines produced secondarily, following the initial cytokine stimulation. For instance, IL-27 induces the production of IFN-γ, which enhances STAT1 activation (Villarino et al., 2003), whereas IL-6 induces IL-21, which promotes phosphorylation of STAT3 (Suto et al., 2008). Obviously, there may be many other factors aside from these two cytokines that may be relevant with respect to the induction of transcriptomes. More studies will be needed to evaluate the range of factors produced in an autocrine manner that contribute to the final output of cell signaling. On a similar note, it will also be interesting to

examine the effect of other factors that engage STAT1 and assess the function of STAT1 as a driver of cytokine specificity for these factors. For example, it will be interesting to compare and contrast effect of type I and type II IFNs with that of IL-27 in terms of cytokine-specific transcriptomic signatures.

In summary, our study provides a quantitative and qualitative evaluation of transcriptional programs elicited by two cytokines that employ highly overlapping signaling pathways and elucidates the molecular basis driving those transcriptome profiles. Despite shared signal mechanisms, the actions of these related cytokines can be remarkably distinct due to combinatorial and asymmetric action of STATs to transcriptomic output and cytokine diversity.

EXPERIMENTAL PROCEDURES

Mice.

C57BL/6J were purchased from Jackson Laboratory, *Stat3*$^{fl/fl}$ mice were from D. Levy (Lee et al., 2002) and bred with CD4-Cre^{+} Tg mice. *Stat1*$^{-/-}$ mice were also from D. Levy (Durbin et al., 1996). All animal studies were performed according to the NIH guidelines for the use and care of live animals and were approved by the Institutional Animal Care and Use Committee of NIAMS.

Naïve CD4^{+} T cell isolation.

CD4^{+} T cells from spleens and lymph nodes of 6–8 week-old mice were purified by negative selection and magnetic separation (Miltenyi Biotec) followed by sorting of naïve CD4^{+}CD62L^{+}CD44^{-}CD25^{-} population using FACSAria II (BD). See supplemental methods for in vitro culture condition and flow cytometry.

Immunoblotting.

Cells were lysed in Triton X-100 lysis buffer containing protease inhibitors. Equal amounts of total protein were separated by PAGE, transferred to nitrocellulose, and blotted with antibodies recognizing actin (Millipore), STAT1 (#9172) or STAT3 (#4904) (Both from Cell Signaling Technology, MA). Secondary antibodies conjugated with IRDye800 (Rockland, Gilbertsville, PA) or Alexa Fluor 680 (Invitrogen) were used for detection, and specific bands were visualized using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

mRNA Sequencing.

Total RNA was prepared from approximately 1 million cells by using mirVana miRNA Isolation Kit (AM1560, ABI). A fraction of total RNA (200 ng) was processed into mRNA-seq library by using TruSeq SR RNA sample prep kit (FC-122–1001, Illumina). The libraries were sequenced for 50 cycles (single read) with a HiSeq 2000 (Illumina). Raw sequencing data were processed with CASAVA 1.8.2 to generate FastQ files, mapped onto the mouse genome build mm9 using TopHat 2.0 (Trapnell and Salzberg, 2009), and gene expression values (RPKM, reads per kilobase exon per million mapped reads) were calculated with Partek Genomics Suite 6.6. All downstream statistical analyses, including

PCA, were performed with Partek Genomics Suite 6.6, R 3.0.1, and GeneSpring GX 12.1 (Agilent Technologies). Differentially regulated genes were selected with following criteria (1) absolute RPKM >1 in at least 1 condition (basal, IL-6- or IL-27-stimulation), and (2) expression change > 2 or < −2.

Gene Set Enrichment Analysis.

Gene set enrichment analysis (GSEA) from the Broad Institute (www.broad.mit.edu/gsea) was used. Th1, Th2 and Th17 cell-associated gene sets were previously published (Roychoudhuri et al., 2013)(GEO# GSE45975).

EMSA.

EMSAs were performed using Gel Shift Assay Systems (Promega). Nuclear extracts were prepared from cultured T cells and incubated at 4°C with ^{32}P-labeled double-stranded oligo (STAT oligo; GATCCGGGAGGGATTTACGGGAAATGCTG) in DNA binding buffer. Electrophoresis was performed on a 4% native polyacrylamide gel (0.5× TBE; acrylamide/bisacrylamide, 29:1), and the radioactivity was visualized by autoradiography.

Circos.

We used Circos (Krzywinski et al., 2009) plots to generate Figure 5A and 5B.

Mosaic plots.

R 3.0.1 was used to generate mosaic plots. For human data sets, cytokine response of each gene was determined and classified into 3 groups (IL-6 unique, IL-27 unique or common) as follows. (1) absolute RPKM >1 in at least 1 condition (basal, IL-6- or IL-27-stimulation), (2) expression fold change > 2 or < −2 by cytokine. Once each gene of each individual was classified for its cytokine specificity, the second round of evaluation was done as a group (healthy control and patient groups separately). (3) IL-6 (or IL-27) unique genes are genes for which in at least 1 subject showed IL-6 (or IL-27) unique response and other subjects were either no response or IL-6 unique. (4) common genes were genes that did not meet the criteria (3).

Human naïve CD4$^+$ T cell isolation.

All samples were collected under approved National Institutes of Health (NIH) protocols; all patients or their parents provided written informed consent. Peripheral blood mononuclear cells were prepared from venous blood by Ficoll-gradient centrifugation. Naïve CD4$^+$CD45RA^{high}CD45RO^{low} population was purified by the flow cytometry using FACSAria II (BD). See supplemental method for in vitro cell culture conditions.

Chromatin immunoprecipitation.

Cells cultured as indicated were cross-linked for 10 minutes with 1% formaldehyde and harvested. Cells were lysed by sonication and immunoprecipitated with indicated antibodies (STAT1 (sc-592, Santa Cruz Biotechnology) or STAT3 (14–6727-81 ebioscience)). Recovered DNA fragments were blunt-end ligated to the Illumina adaptors, amplified, and sequenced by using the Hi-Seq 2000 (Illumina, San Diego, CA). Reads of 50 bases were

aligned to the mouse genome build mm9 with Bowtie 0.12.8 (Langmead et al., 2009), allowing two mismatches. Uniquely mapped and non-redundant reads were used for peak calling using MACS 1.4.2 (Zhang et al., 2008) with a p-value < 1.0E-05.

STAT binding box plots.

Following peak calling by MACS 1.4.2, PAPST (Bible et al. 2015) was used to calculate the combined peak reads (normalized to per million) within extended gene body (RefGene, from (TSS-50k) to (TTS+50k)). The log2 transformed gene body reads were used to create boxplots, each with data corresponding to a set of genes of interest under a specific biological condition. The log2 transformed read data for boxplots were also used for p-value calculations using Welch t-test. Both boxplots and p-values (Supplemental Table 1) were generated using R.

Statistics.

For statistical analysis, Student's *t*-test was used unless otherwise specified to calculate statistical significance of the difference in mean values and *P* values. For calculation of correlation coefficient, Pearson's correlation was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The transcriptomic output driven by IL-6 or IL-27 is primarily regulated by STAT3
- STAT1 does not compensate for STAT3 to drive transcriptional output
- Much of STAT1 binding to chromatin is STAT3 dependent
- The ability to access STAT1 magnifies the difference between IL-6 and IL-27

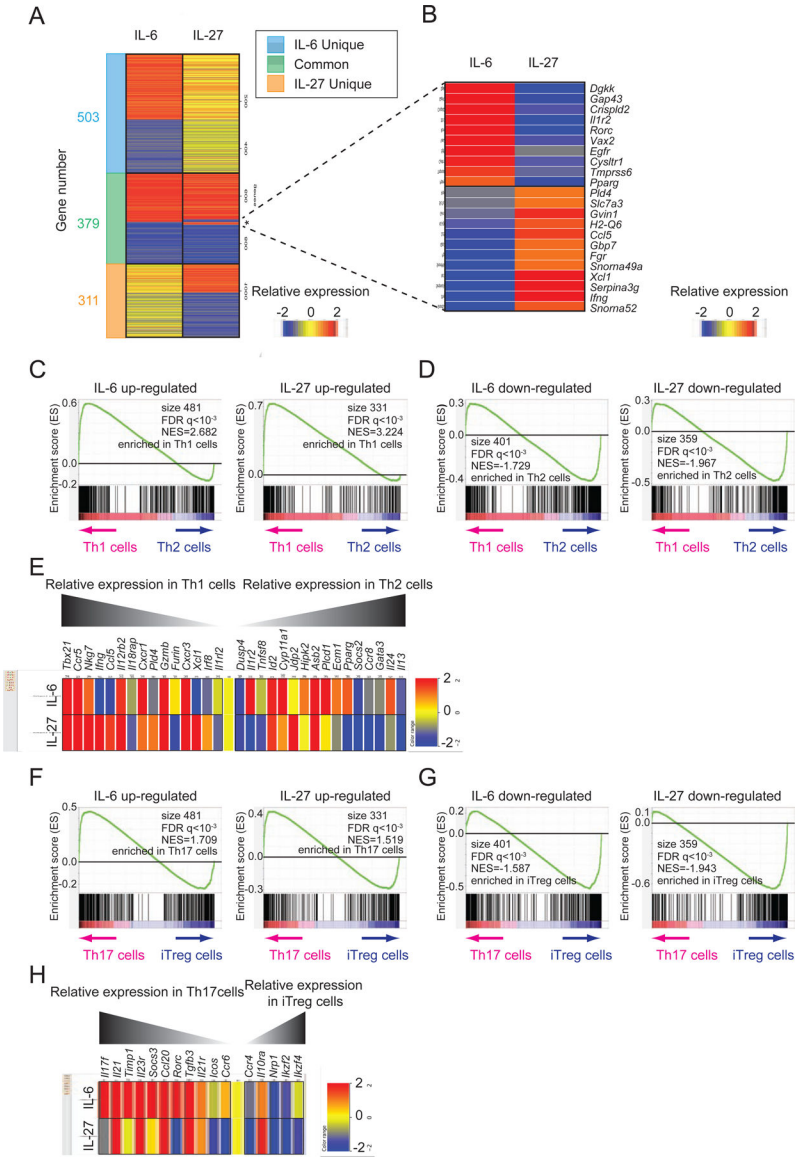

Figure 1. Quantitation and analysis of IL-6 and IL-27 transcriptomic responses

Naïve CD4$^+$ T cells were cultured for 3 days on anti-CD3 and anti-CD28 coated plates with or without IL-6 or IL-27. Gene expression was measured by RNA-seq as RPKM (reads per kilobase exon model per million reads). (**A**) Gene expression changes of >2 fold or <0.5 fold by IL-6 or IL-27 compared to cells cultured without exogenous cytokines (n=1193) are depicted by heat-map and clustered based on their selective regulation by IL-6 (IL-6 Unique), IL-27 (IL-27 Unique) or both cytokines (Common). The small subset of atypical genes, oppositely regulated by IL-6 and IL-27, are denoted by the asterisk and are highlighted in (**B**). (**C, D**) Gene Set Enrichment Analysis (GSEA) for IL-6- or IL-27-regulated genes are compared to genes expressed in Th1 and Th2 cells (data from GEO# GSE45975). IL-6 up-regulated, IL-27 up-regulated, IL-6 down-regulated, IL-27 down-regulated genes are plotted separately, and each analysis shows non-random distribution of IL-6- or IL-27-regulated genes versus Th1 or Th2 cell-associated genes (FDR; false

discovery rate, NES; normalized enrichment score). (**E**) IL-6- and IL-27-dependency of representative Th1 and Th2 cell-associated genes identified by GSEA analysis in C and D are shown. (**F, G**) GSEA for IL-6 or IL-27 regulated-gene sets plotted against genes expressed in Th17 and iTreg cells (data from GEO# GSE45975). (**H**) IL-6 and IL-27 dependency of representative Th17- and iTreg cell-expressed genes. The RNA-seq data were acquired as biological duplicates, and the average of the two was used for all downstream analyses presented in Figures.1, 3, 4 and 5. See also Supplemental Figure 1.

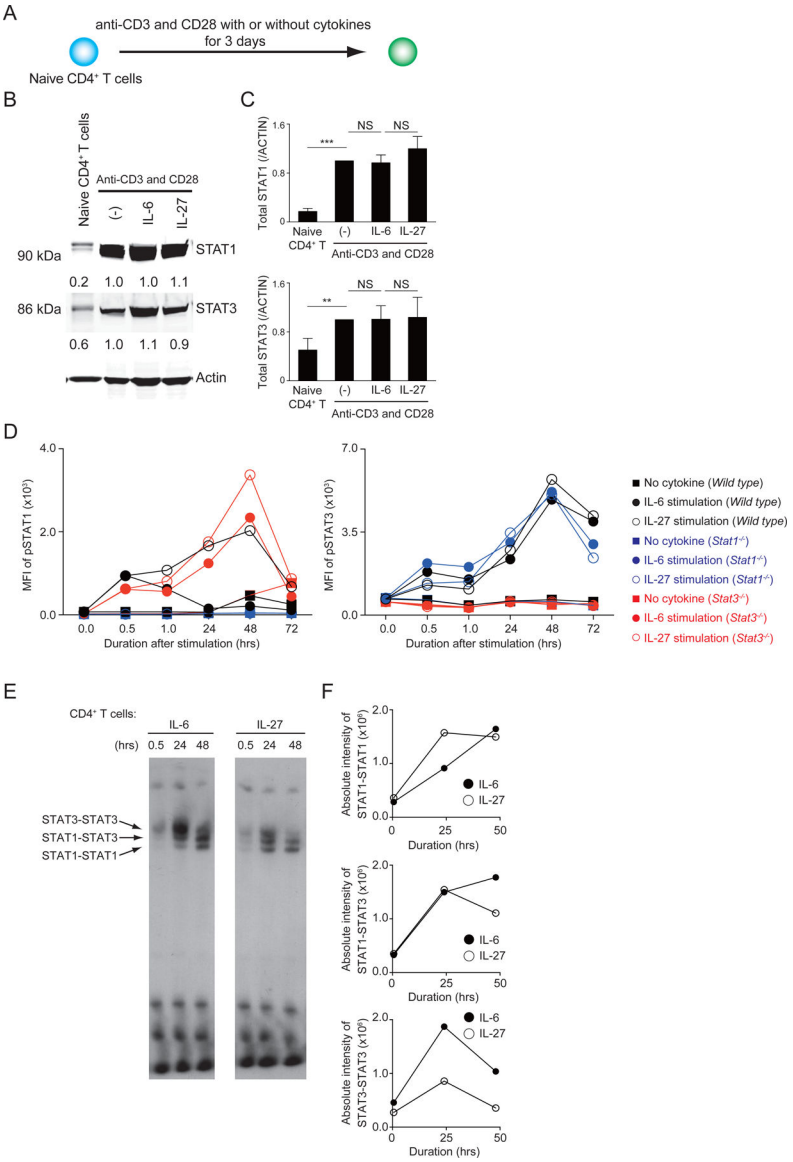

Figure 2. The ability of IL-6 and IL-27 to access STAT1 and STAT3 dynamically changes with T cell activation.

Sorted naïve CD4$^+$ T cells were stimulated with anti-CD3 and anti-CCD28 with or without the indicated cytokines for 3 days (**A**). With indicated stimulation and time points, STAT1 and STAT3 protein amounts were measured by immunoblotting (**B, C**), intracellular staining and flow cytometry (phospho-STATs) (**D**) and EMSA (**E, F**). (**C**) Pooled data from 4 independent experiments are provided (***P<0.001, **P<0.01, NS, not significant). (**D**) Time course of STAT1 and STAT3 phosphorylation following cytokine stimulation was evaluated for up to 72 hours. (**E**) The positions of STAT1-STAT1, STAT1-STAT3 and STAT3-STAT3 dimers are indicated. (**F**) The intensity of STAT3-STAT3, STAT3-STAT1 and STAT1-STAT1 dimer species in (**E**) was quantitated by densitometry. See also Supplemental Figure 2.

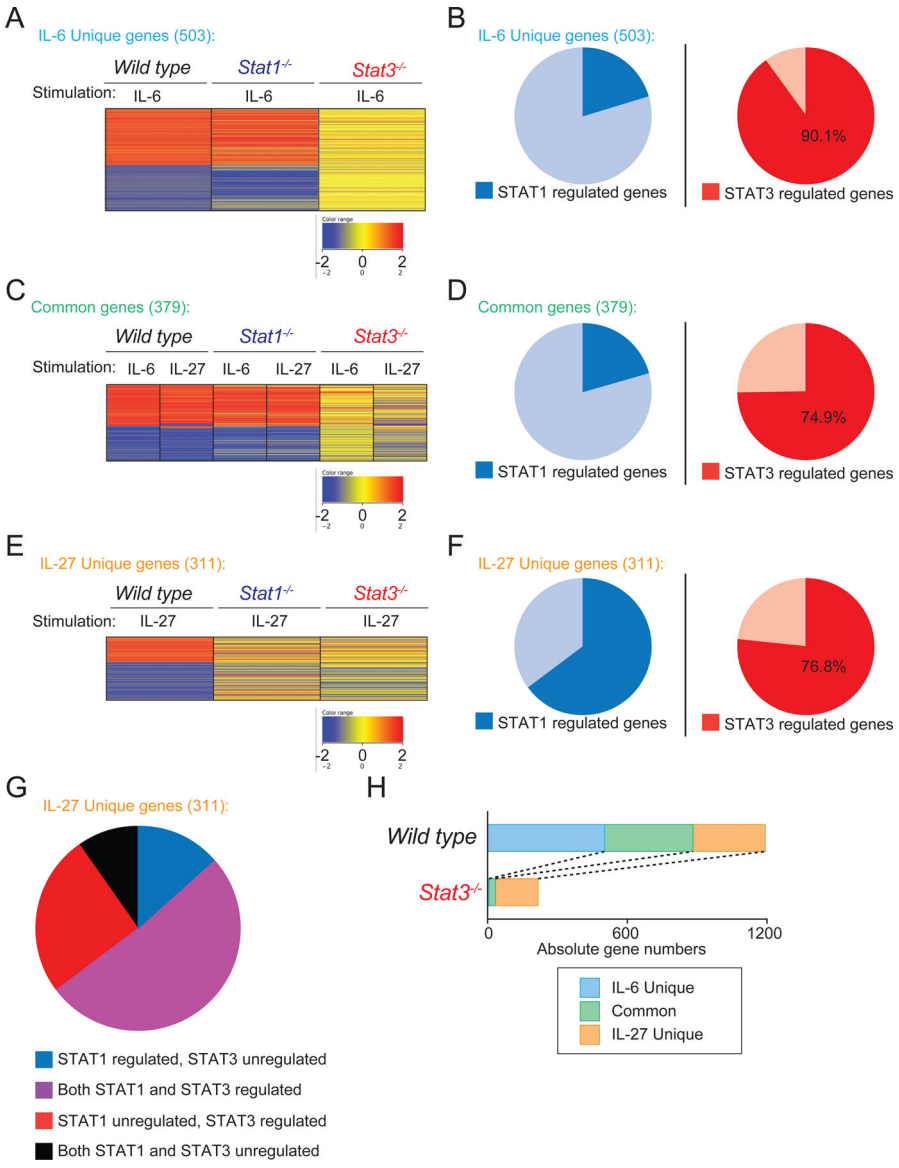

Figure 3. STAT3 controls the magnitude of transcriptional output induced by both IL-6 and IL-27.

Naïve CD4$^+$ T cells from wild type, *Stat1*$^{-/-}$ or *Stat3*$^{-/-}$ mice were cultured on anti-CD3/CD28 coated plates with or without IL-6 or IL-27 for 3 days. RNA was isolated and global gene expression was determined by RNA-Seq. (**A, C, E)**, A group of cytokine responsive genes was selected similarly as in Figure 1A. Fold changes in expression of genes selectively regulated by IL-6 (**A**, n=503 genes) and IL-27 (**E**, n=311 genes) or both cytokines (**C**, n=379 genes) in wild type, STAT1- or STAT3-deficient CD4$^+$ T cells are shown by heat-maps. (**B, D, F**) STAT dependency was evaluated based on the loss of gene regulation in *Stat1*$^{-/-}$ or *Stat3*$^{-/-}$ CD4$^+$ T cells and is depicted by pie charts. (**G**) Contribution of STAT1, STAT3 or both in controlling genes selectively regulated by IL-27. (**H**) Consequence of absence of STAT3 in mouse CD4$^+$ T cells on genes regulated by IL-6 (blue), IL-27 (orange) and both cytokines (green). See also Supplemental Figure 3.

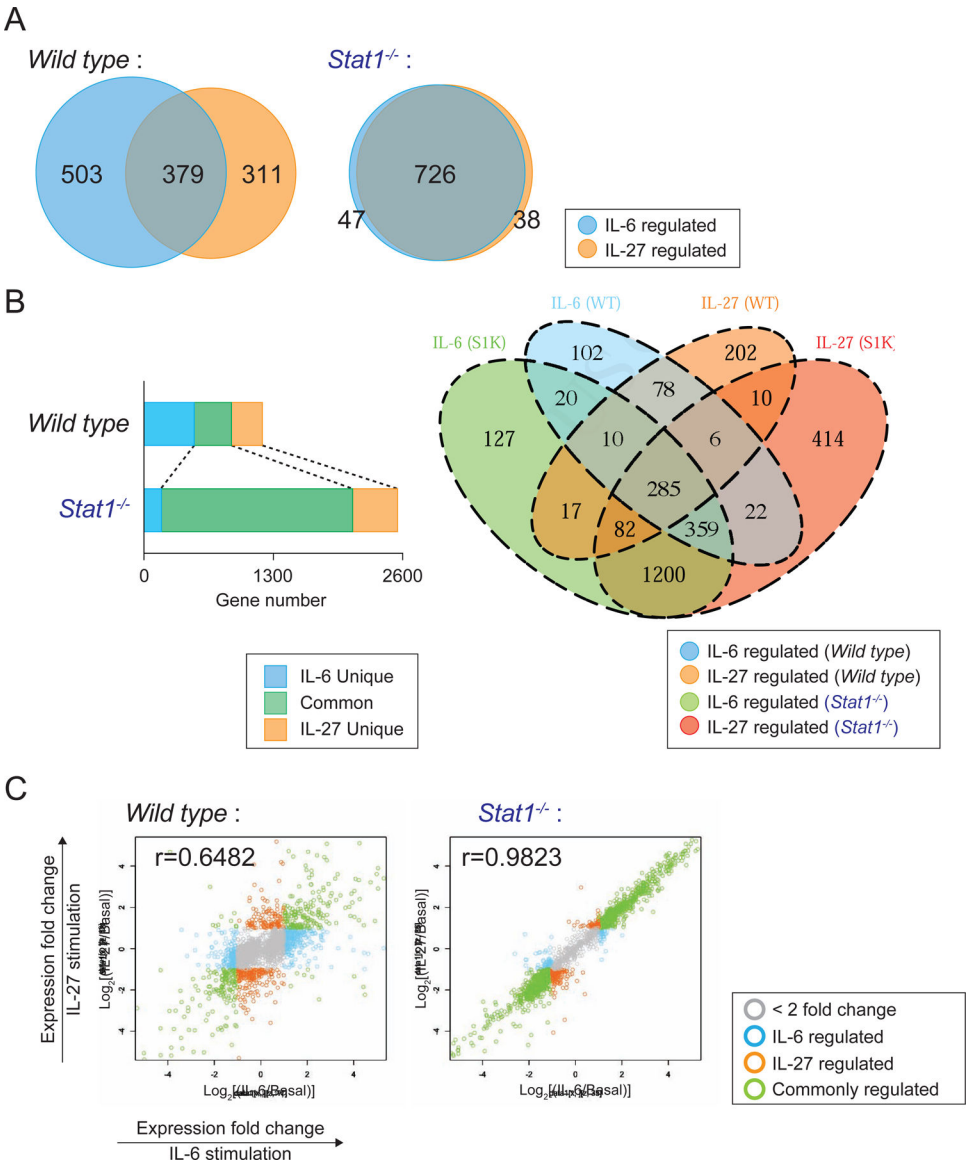

Figure 4. STAT1 controls the diversity of cytokine responses mediated by IL-6 and IL-27.

Wild type and STAT1-deficient naïve $CD4^+$ T cells were activated and cultured with cytokines. (**A**) A total of 1193 genes were depicted in wild type cells as regulated by IL-6 (blue) or IL-27 (orange) and their gene expression overlap was depicted for wild type and *Stat1*$^{-/-}$ cells (**B**) The number of genes regulated by IL-6 or IL-27 was increased in *Stat1*$^{-/-}$ cells. These genes were further classified into subgroups based on their overlapping or unique cytokine response. Many genes acquired cytokine response in *Stat1*$^{-/-}$ indicative of *de novo* gene regulation in *Stat1*$^{-/-}$ (green and dark orange). (**C**) Genes regulated by IL-6 (blue), IL-27 (orange), both (green) or neither (gray) are identified and color-coded. The scatter plots are drawn to evaluate the similarity of gene expression between IL-6 (X axis) and IL-27 (Y axis) and the correlation coefficient (r) was calculated. See also Supplemental Figure 4.

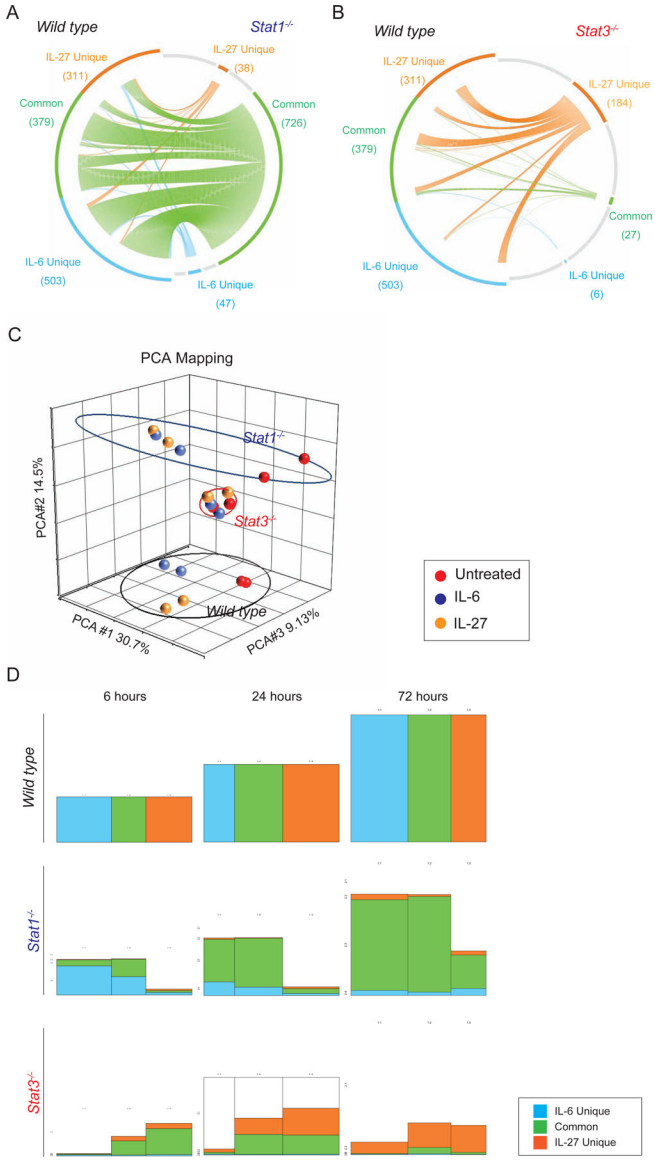

Figure 5. Asymmetric contribution of STAT3 and STAT1 in driving magnitude and specificity of gp130 cytokine responses.

(**A, B**) Circos visualization to show the consequence of absence of STAT1 (**A**) or STAT3 (**B**) on cytokine regulated gene expression change. Cytokine response of genes was color coded as IL-6 unique (blue), common (green), or IL-27 unique (orange) subgroups. Genes in wild type cells were aligned on the left side of the plots and the genes in STAT-deficient cells were aligned on the right side of the plots with each connecting line link the same gene on left and right. The color of connecting line represented cytokine specificity of STAT-deficient cells. The genes that lost cytokine responsiveness in *Stat1*$^{-/-}$ or *Stat3*$^{-/-}$ were shown in gray lines on the right half of the circle without connecting lines. Many IL-6 unique or IL-27 unique genes in wild type switched to common genes in *Stat1*$^{-/-}$. Notable fraction of IL-6 unique or common genes in wild type became IL-27 unique genes in *Stat3*$^{-/-}$. (**C**) Principal component analysis of the global gene expression is shown as clusters are grouped by genotype (wild type, *Stat1*$^{-/-}$, *Stat3*$^{-/-}$). A total of 18 samples (9 conditions

in duplicate) of RNA-seq data were plotted. The three main principal components in the model contribute to explain 30.7%, 14.5% and 9.13% of the variation, respectively, and are predictive. (**D**) The relative numbers of genes regulated by IL-6 (blue), IL-27 (orange) or both cytokines (green) are depicted as areas of the different rectangles in mosaic plots for the indicated time points.

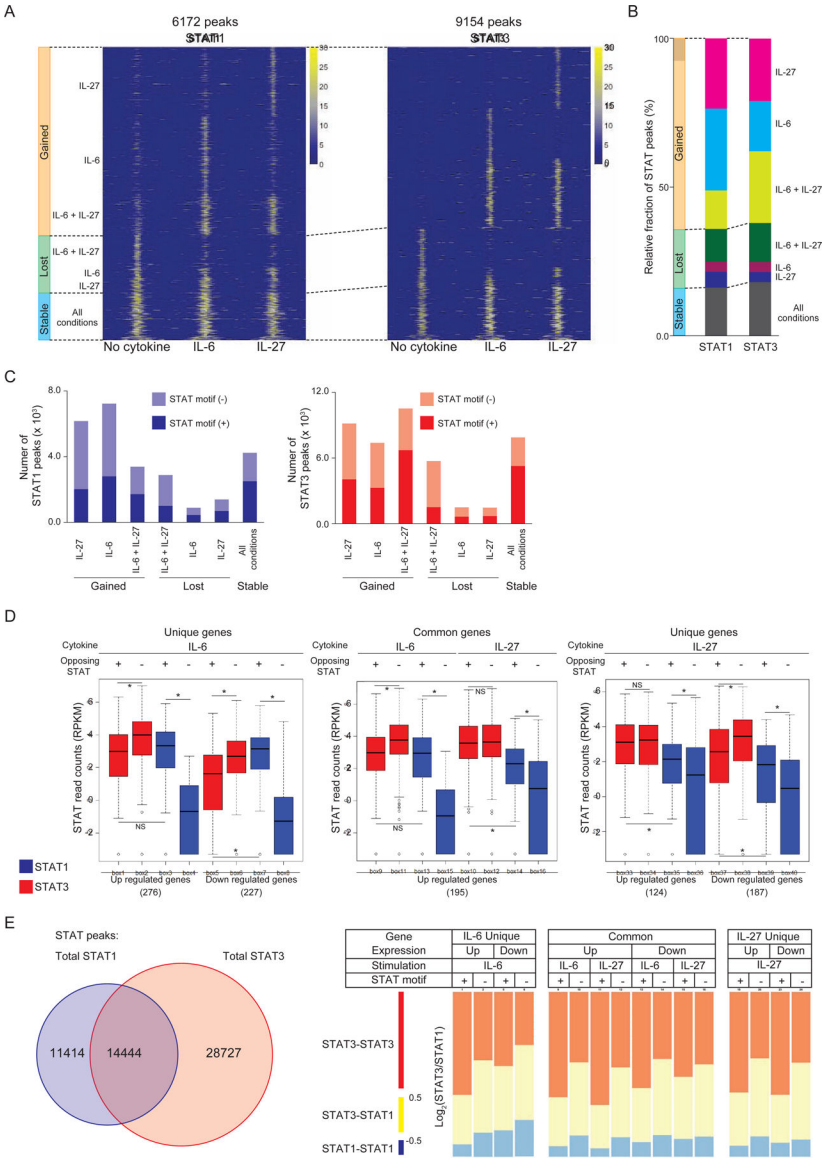

Figure 6. STAT1 binding to chromatin is largely STAT3 dependent.

(**A**) STAT1 (right) and STAT3 (left) binding was assessed by ChIP-seq and clustered into 7 categories based on cytokine response to IL-6 and IL-27 as indicated. (**B**) Proportions of peaks segregating into the clusters shown in panel A were shown for STAT1 and STAT3. (**C**) Quantitation of STAT1 (blue) and STAT3 (red) peaks with or without a STAT-binding motif was shown for 7 categories. (**D**) STAT3 (red) and STAT1 (blue) read counts within 50 kb of TSS and TES of cytokine regulated genes in wild type and STAT-deficient cells. The number of genes in each group was shown in a parenthesis. See Methods (stat binding box plots) for details. (*P<0.05, NS, not significant). (**E**) The numbers of overlapping and unique peaks bound by STAT1 and STAT3 are shown in Venn diagrams. The potential distinct STAT dimer composition was determined by assessing the ratio of STAT3 and STAT1 signals and linked to the nearest gene. The STAT peaks were then sorted based on the cytokine response

of the nearest genes. STAT1 and STAT3 ChIP-Seq experiments were done 2 times under similar conditions. See also Supplemental Figure 5 and Table 1.

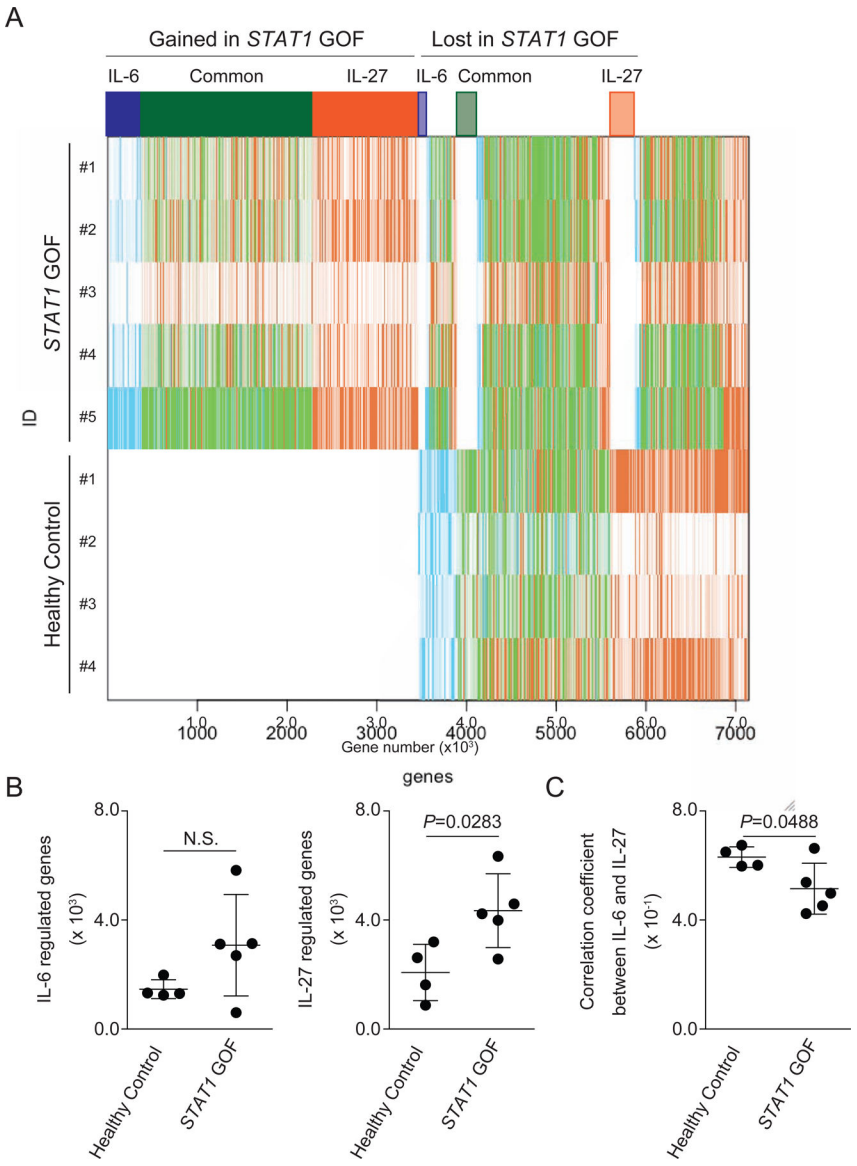

Figure 7. STAT1 gain-of-function mutations in human CD4+ T cells exhibit enhanced transcriptomic output and diversity in response to IL-6 and IL-27.

Sorted naïve $CD4^+$ T cells ($CD4^+CD45RA^+$) from age- and sex-matched healthy control (HC) and patients with STAT1 gain-of-function (*STAT1* GOF) (Table S2) were stimulated with plate-coated anti-CD3 and anti-CD28 with or without (untreated) IL-6 (50 ng ml^{-1}) or IL-27 (50 ng ml^{-1}) for 3 days. RNA was isolated and analyzed by mRNA-Seq. (**A**) Genes whose expression was altered are clustered based on their selective regulation by IL-6 (blue), IL-27 (orange) or commonly (green) by both cytokines in healthy control (HC) subjects (n=4) and *STAT1* GOF patients (*STAT1* GOF) (n=5). See Methods (mosaic plots) for details. (**B**) The absolute number of cytokine regulated genes was quantitated (mean ± s.e.m., *P* value by unpaired student's t test, NS, not significant). (**C**) Similarities in IL-6 and IL-27-regulated genes were quantitated (Pearson correlation coefficients, mean ± s.e.m., *P* value by unpaired student's t test). See also Supplemental Table 2 and 3.