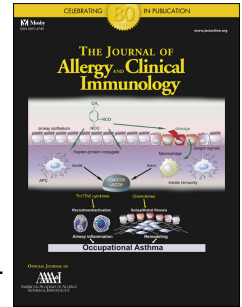


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Elucidating the effects of disease-causing mutations on STAT3 function in autosomal dominant hyper-IgE syndrome

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Key words

• autosomal dominant hyper-IgE syndrome; STAT3; transcription factors; cytokine signaling

Abbreviations

AD-HIES: autosomal dominant hyper-IgE syndrome; DN: dominant negative; JAK: Janus activating kinase; STAT: signal transducer and activation of transcription; SH2: Src homology 2; DNA BD: DNA binding domain; TA: transactivation; EBV-LCL: EBV-transformed lymphoblastoid cell line; WT: wild-type

Capsule summary

Analysis of germline mutations in *STAT3* that cause AD-HIES revealed biochemical mechanisms of disease-causation. This has implications for developing therapeutics that could modulate the function of STAT3 in the settings of immunodeficiency, autoimmunity and malignancy.

To the editor

Signaling through STAT3 is an important process required for the function of a wide range of biological systems. This is demonstrated by embryonic lethality of germline *Stat3* gene-targeted mice¹. Furthermore, heterozygous dominant negative (DN) mutations in *STAT3* cause autosomal dominant hyper IgE syndrome (AD-HIES) in humans, a disease characterized by increased susceptibility to infection with *Staphylococcus aureus*, *Streptococcus pneumonia* and *Candida albicans*, eczema, extremely elevated levels of serum IgE, poor humoral immune responses, and non-immunological features including defects in musculoskeletal, dental, and vascular systems.^{2, 3} More than 60 different mutations have been reported that affect all functional domains of STAT3.^{3, 4} *STAT3* encodes a latent cytosolic transcription factor with a key role in signaling downstream of numerous cytokines, including IL-6, IL-10, IL-21 and IL-23². DN *STAT3* mutations cause multiple cellular defects, including reduced Th17 cells, memory B cells, T follicular helper cells and MAIT cells, and impairing responsiveness of naïve B cells to IL-10 and IL-21 (Online Supplemental data Table 1)^{2, 3, 5, 6}. These defects explain susceptibility to fungal infections and poor antibody production in AD-HIES. Despite this, our understanding of the molecular impact of various *STAT3* mutations remains limited. Indeed, as there is no genotype/phenotype relationship⁷, it remains to be determined how diverse mutations impair STAT3 signaling to manifest the same clinical and cellular phenotype. Furthermore, while several studies have examined the effects of mutations in separate domains of STAT3 on signaling pathways⁸⁻¹⁰, few have systematically compared the impact of mutations located in different functional domains of STAT3.

To explore this, we examined the consequences of distinct disease-causing mutations in the DNA binding, SH2 and transactivation domains on each stage of STAT3 signaling. We selected 6 mutations previously identified in numerous cohorts of AD-HIES patients, including our own, that localize to the DNA binding domain (BD) (R382W, H437P, S465F), SH2 domain (Q644P, Y657N) or TA (L706M) domains^{5, 6} (Figure 1A; Fig S1A Online supplemental data). All mutations have a comparable deleterious effect on the generation of Th17, Tfh and memory B cells in affected individuals (Online Supplemental data Table S1). To test how each mutation affected tyrosine phosphorylation, HEK-293T cells were transfected with plasmids encoding WT or mutant STAT3 (Figure 1B, Fig S1B). IL-6 stimulation induced phosphorylation at tyrosine 705 (Y705) of WT STAT3 as well as STAT3 harboring mutations in the DNA BD, as well as the Q644P SH2 domain mutant (Figure 1B, Fig S1B). In contrast, the SH2 domain Y657N and TA domain L706M mutants failed to undergo significant phosphorylation in response to IL-6 (Figure 1B, Fig S1B). Reduced STAT3 phosphorylation

80 due to Y657N SH2 and L706M TA domain mutants likely results from conformational
81 alterations of STAT3 or steric hindrance that limit access of kinases to phosphorylate Y705.
82 Thus, only mutations in the proximity of Y705 (Figure 1A) affected STAT3 phosphorylation.

83
84 Following phosphorylation, STAT3 forms dimers that are required for function^{11, 12}. To test the
85 ability of mutant STAT3 to form dimers with WT STAT3, and to recapitulate the setting of
86 heterozygous *STAT3* mutations in AD-HIES, HEK-293T cells were co-transfected with Myc-
87 tagged WT and Flag-tagged mutant STAT3 plasmids (Figure 1C), and then stimulated with IL-
88 6. STAT3 multimers were captured by co-immunoprecipitation using anti-Flag mAb. The
89 levels of dimer formation between WT STAT3 and those with mutations in the DNA BD or TA
90 domain were comparable to WT/WT STAT3 proteins (Figure 1C, D). This is consistent with
91 initial observations by Minegishi et al.¹⁰ who reported that mutations in the DNA BD did not
92 affect STAT3 dimerization. In contrast to DNA BD mutations, SH2 domain mutations reduced
93 dimer formation by ~40-50%, with Y657N having the greatest effect (Figure 1C,D). This
94 identifies these residues as being critical for intermolecular interactions between
95 phosphorylated STAT3 (pSTAT3). It is likely that amino acid substitutions in the SH2 domain
96 result in changes in charge, which affect assembly or stability of STAT3 dimers.

97
98 Nuclear translocation of STAT3 is crucial for its role as a transcription factor^{11, 12}. To
99 investigate the impact of mutations in *STAT3* on its nuclear translocation, nuclear extracts
100 were prepared from IL-21-stimulated WT and STAT3 mutant EBV-LCLs and the levels of
101 pSTAT3 determined. The accumulation of nuclear pSTAT3 in normal EBV-LCLs was maximal
102 after 15 mins and declined at times thereafter (Figure 2A). Consistent with reductions in total
103 pSTAT3 in transfected HEK-293T cells (Figure 1B; Fig S1B), the Y657N SH2 and L706M TA
104 domain mutations resulted in significant reductions in levels of nuclear pSTAT3 in EBV-LCLs
105 (Figure 2A). Remarkably, two of the three DNA BD mutations (R382W, H437P) significantly
106 reduced nuclear translocation of pSTAT3 (Figure 2A). As several nuclear export sequences
107 have been identified in the DNA BD of STAT3¹³, reduced translocation due to these mutations
108 this likely results from an imbalance between nuclear import and export. Interestingly, the
109 S465F DNA BD mutation had no effect on nuclear pSTAT3 translocation (Figure 2A).

110
111 Once in the nucleus STAT3 binds specific target sites to regulate gene transcription.^{11, 12} WT
112 STAT3 exhibited strong binding to a canonical target sequence that was maximal after 15-30
113 mins of IL-21 stimulation (Figure 2B). Conversely, all *STAT3* mutant proteins showed
114 significant decreases in their ability to bind DNA (Figure 2B). As some *STAT3* mutations
115 affected phosphorylation (Y657N, L706M) or nuclear translocation (R382W, H437P), the

reduction in DNA binding reflects not only an impaired ability of mutant STAT3 to bind target sequences but also decreases in the amount of pSTAT3 in the nucleus. Consistent with these findings, induction of the STAT3 target genes *SOCS3* (Figure 2C), *PRDM1* (encoding BLIMP-1) and *IL2RA* (Fig S2A, B) were significantly reduced (50-90%) in response to IL-21 and IL-10 in *STAT3* mutant compared to normal EBV-LCLs, thereby indicating that the defect in DNA binding results in a functional impairment in the ability to induce gene transcription. Thus, impaired DNA binding of mutant STAT3 proteins manifests as reduced gene transcription.

In conclusion our study gives insight into dysfunctional STAT3-dependent signaling in AD-HIES, and allowed for the dissection and identification of specific stages of STAT3 signaling affected by these disease-causing mutations (Fig S3; Table S1). This not only highlights which aspects of STAT3 signaling are impacted by these mutations but also identifies residues and regions of domains in STAT3 that are critical for normal function (Fig S3; Table S1). This reveals mechanisms not only for loss-of function of STAT3 in AD-HIES but reveals residues/regions of STAT3 that could be targeted in cases where STAT3 is overactive, such as gain-of function mutations in *STAT3* resulting in multisystemic autoimmunity or hematological malignancies, respectively³.

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Figure Legends**Figure 1. Autosomal dominant hyper IgE syndrome-causing *STAT3* mutations: effects on phosphorylation and dimerisation.**

(A) Schematic representation of *STAT3*, with the mutations studied indicated by black lines. (B) HEK-293T cells were transfected with Flag-tagged WT or R382W, H437P, S465F, Q644P, Y657N or L706M mutant *STAT3*, and either untreated or stimulated with IL-6 (30 mins). Phosphorylation of *STAT3* (p*STAT3*) was determined by fixing and permeabilising the cells and labeling with Abs specific for Flag and p*STAT3* (Y705). Graph shows geometric MFI of p*STAT3* (Two way ANOVA with Dunnett post-test, comparison between IL-6 stimulated WT and mutant *STAT3*. *** $p < 0.001$. mean $\pm$ SEM, $n=6$). (C, D) HEK-293T cells were co-transfected with plasmids containing Myc-tagged WT *STAT3* and either Flag-tagged WT or mutant *STAT3*. After 48 hours, cells were harvested, rested for 2 hours then stimulated with IL-6 (10 ng/ml, 30 mins). Cell lysates were immunoprecipitated with anti-Flag Ab, and assessed for the presence of Myc-tagged WT or mutant *STAT3* by Western blotting. (C) Diagram of the gel lanes (upper); Input whole cell lysates immunoblotted for Myc, Flag or GAPDH showing equivalent amounts of WT and mutant *STAT3* (left; lower) and immunoprecipitation of lysates with anti-Flag beads, immunoblotted for Myc and Flag (right; lower). (D) Quantification of ratio of WT:mutant *STAT3*, normalized to WT:mutant ratio of the input. (One way ANOVA with Dunnett post-test, comparison between WT and various mutants. ** $p < 0.01$, * $p < 0.05$. mean $\pm$ SEM, $n=5$).

Figure 2. Impaired nuclear translocation, DNA binding and gene induction by mutant *STAT3* proteins.

(A, B) EBV-LCLs from normal donors or AD-HIES patients were stimulated with IL-21 for 0, 15, 30 or 60 minutes, and nuclear fractions then isolated. (A) The abundance of p*STAT3* was assessed by determining the ratio of intensity of mutant vs WT p*STAT3* stimulated with IL-21 for 15 minutes. (Two way ANOVA with Dunnett post-test, comparison between 15 minute time points, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. mean $\pm$ SEM, $n=4$). (B) Binding of WT and mutant *STAT3* to consensus DNA binding sites was determined (TransAM kit, ActiveMotif). Values were normalized to WT *STAT3* at 15 mins. (Two way ANOVA with Dunnett post-test, comparison between 15 minute time points, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. mean $\pm$ SEM, $n=3-4$). The dashed line represents the basal response (i.e. time 0) of WT EBV LCLs to cytokine stimulation. (C) EBV-LCLs from normal donors or AD-HIES patients were stimulated with IL-21 or media only for 3 hours. Expression levels of *SOCS3* were determined by qPCR. (Two way ANOVA with Holm-Sidak post-test, comparison between IL-21 stimulated (*SOCS3*) samples, ** $P < 0.01$, * $P < 0.05$. mean $\pm$ SEM, $n=5-9$).

Supplemental Online data

Figure S1: Autosomal dominant hyper IgE syndrome-causing *STAT3* mutations and effects on cytokine-induced phosphorylation

(A) Crystallographic structure of a *STAT3* dimer bound to DNA (Protein Data Bank, 1BG1). Sites of the mutations are represented by red spheres, phosphorylated tyrosine 705 (Y705) is indicated by black spheres.

(B) HEK-293T cells were transfected with Flag-tagged WT *STAT3* or *STAT3* harbouring the following mutations: R382W, H437P, S465F, Q644P, Y657N or L706M. Cells were left untreated or stimulated with IL-6 for 30 mins. Phosphorylation of *STAT3* was determined by flow cytometry following fixing and permeabilising the cells and labeling with Abs specific for Flag and p*STAT3* (Y705). Representative histograms of phosphorylated *STAT3* induction after IL-6 stimulation.

Figure S2: Effect of *STAT3* loss-of function mutations on induction of expression of target genes.

EBV-LCLs from normal donors or AD-HIES patients were unstimulated or stimulated with IL-10 (A, B), IL-21 (C) for 3 hours. Expression of *PRDM1* and *IL2RA* was then determined by qPCR (Two way ANOVA with Holm-Sidak post-test, comparison between treated and untreated (*PRDM1* and *IL2RA*), * $P < 0.05$. mean $\pm$ SEM, n=5-9)

Figure S3: Summary of the proximal stage of signaling disrupted by various AD-HIES *STAT3* mutations. A diagram showing the stages of signaling affected by each *STAT3* mutation. IL-21 bound to IL-21R (Protein Data Bank, 3TXG) used as an example of a *STAT3* signaling cytokine receptor. Phosphorylation of tyrosine 705 (pY705) is represented in the *STAT3* structure with black spheres (Protein Data Bank, 1BG1). Orange box surrounding the mutation indicates TA domain, yellow box indicates SH2 domain and green box indicates DNA binding domain.

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Figure 1

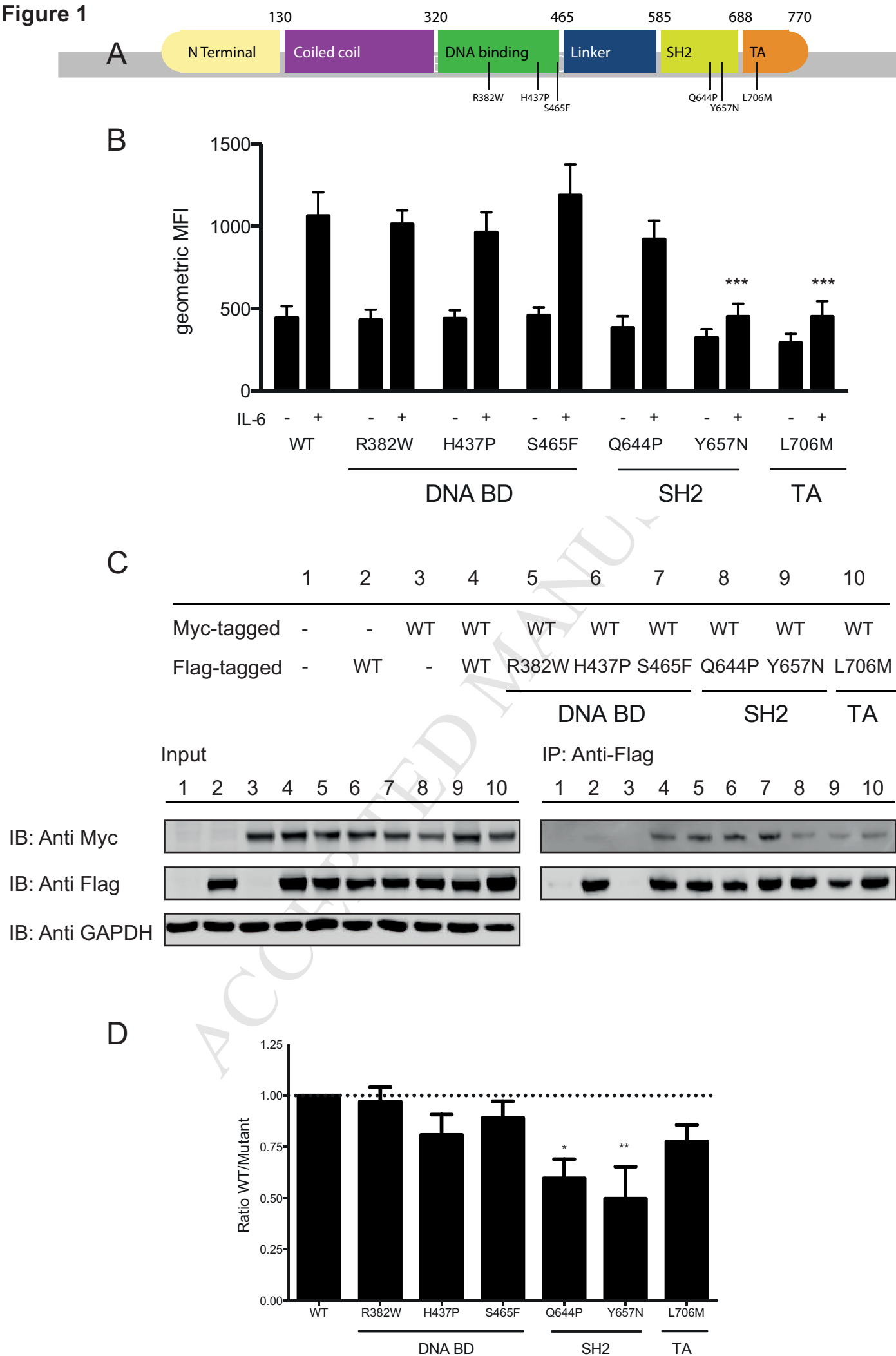


Figure 2

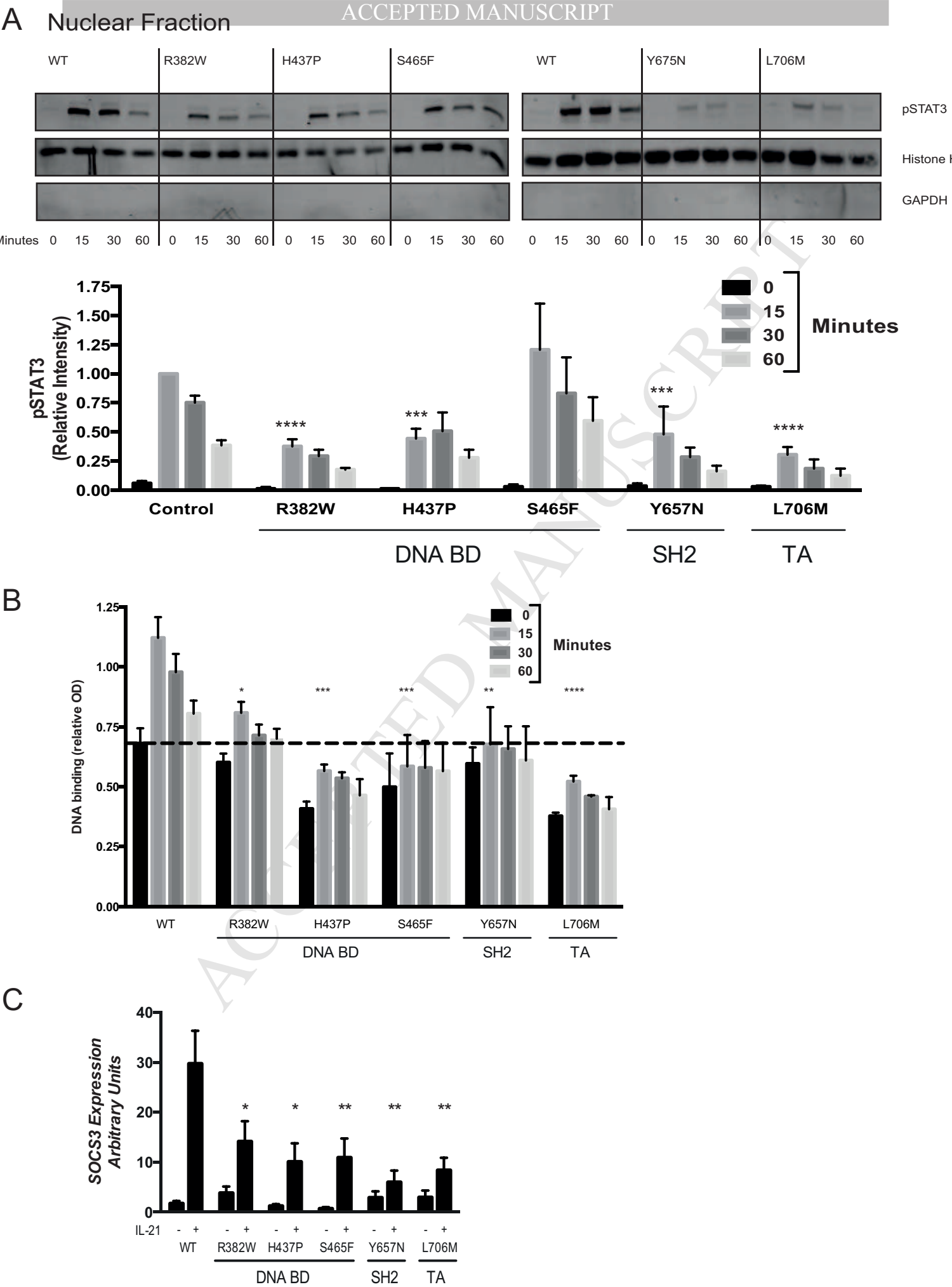


Table S1: Effects of STAT3 mutations on the generation of effector lymphocytes and on STAT3 signaling and function

Lymphocyte population/function	STAT3 mutation					
	R382W	H437P	S465F	Q644P	Y657N	L706M
Th17 cells (%CD45RO ⁺ CCR6 ⁺ CD4 ⁺ T cells); <i>Reference values: 23.0 ± 8.7% (mean ± sd; n=68)</i>	3.55	10	3.3	5.5	4	7.5
% Memory B cells (%CD27 ⁺ CD20 ⁺ cells) <i>Reference values: 27.5 ± 14.5% (mean ± sd; n=64)</i>	5.3	3.5	1.3	4.2	2.1	7.8
Tfh cells (%CD45RA ⁺ CXCR5 ⁺ CD4 ⁺ T cells) <i>Reference values: 8.2 ± 3.3% (mean ± sd; n=72)</i>	3.8	3.5	4.5	3.7	2.5	2.5
Biochemical feature of STAT3 signaling	R382W	H437P	S465F	Q644P	Y657N	L706M
Phosphorylation	Normal	Normal	Normal	Normal	↓	↓
Dimerisation	Normal	Normal	Normal	↓	↓	Normal
Nuclear localization	↓	↓	Normal	ND	↓	↓
DNA binding	↓	↓	↓	ND	↓	↓
Induction of target genes	↓	↓	↓	ND	↓	↓

The proportions of Th17 cells, memory B cells, and T follicular helper (Tfh) cells in the peripheral blood of individual(s) with each mutation were determined by flow cytometry. For the R382W and L706M mutations, the values represent the mean from analysis of several patients with the same mutation. For the H437P, Q644P and Y657N mutations the values represent the mean of repeated analyses on the same individual. Reference ranges were from our previously published studies¹⁻⁴. The effect of each of the mutations tested on the distinct stages of the STAT3 signaling pathway is also indicated.

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Figure S1

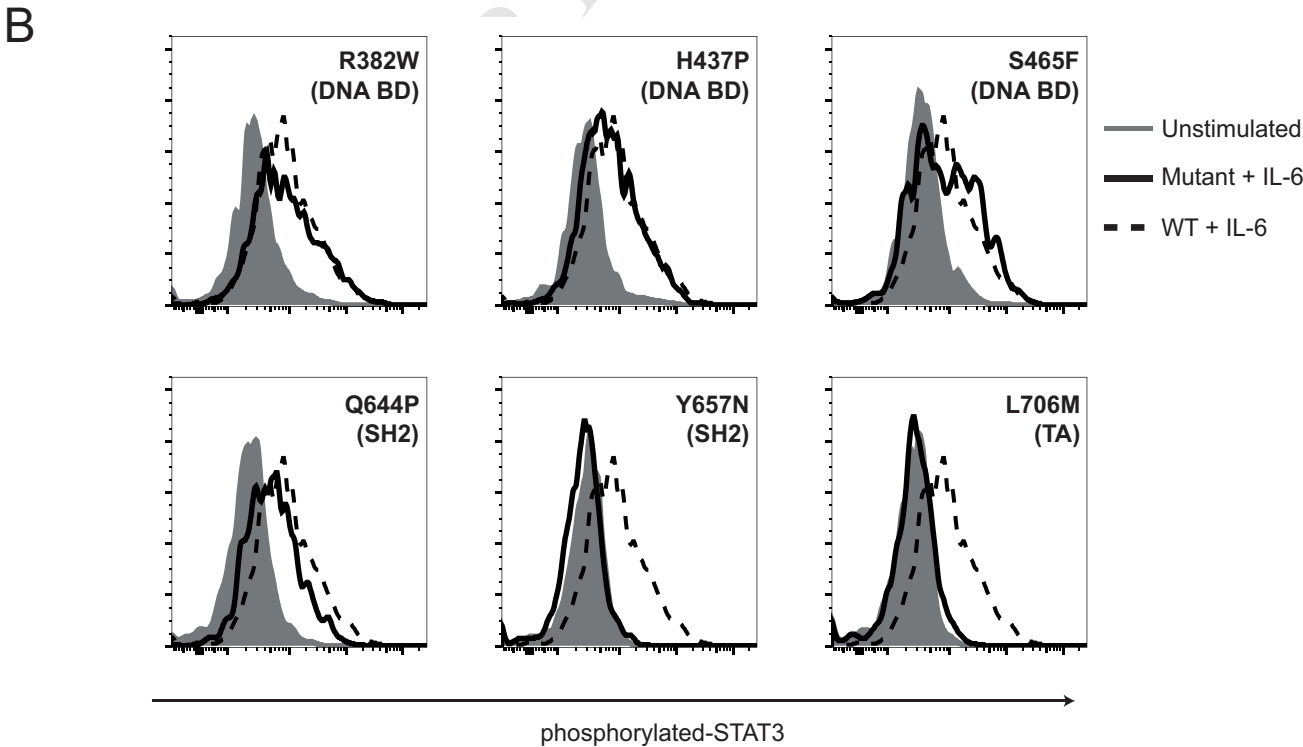
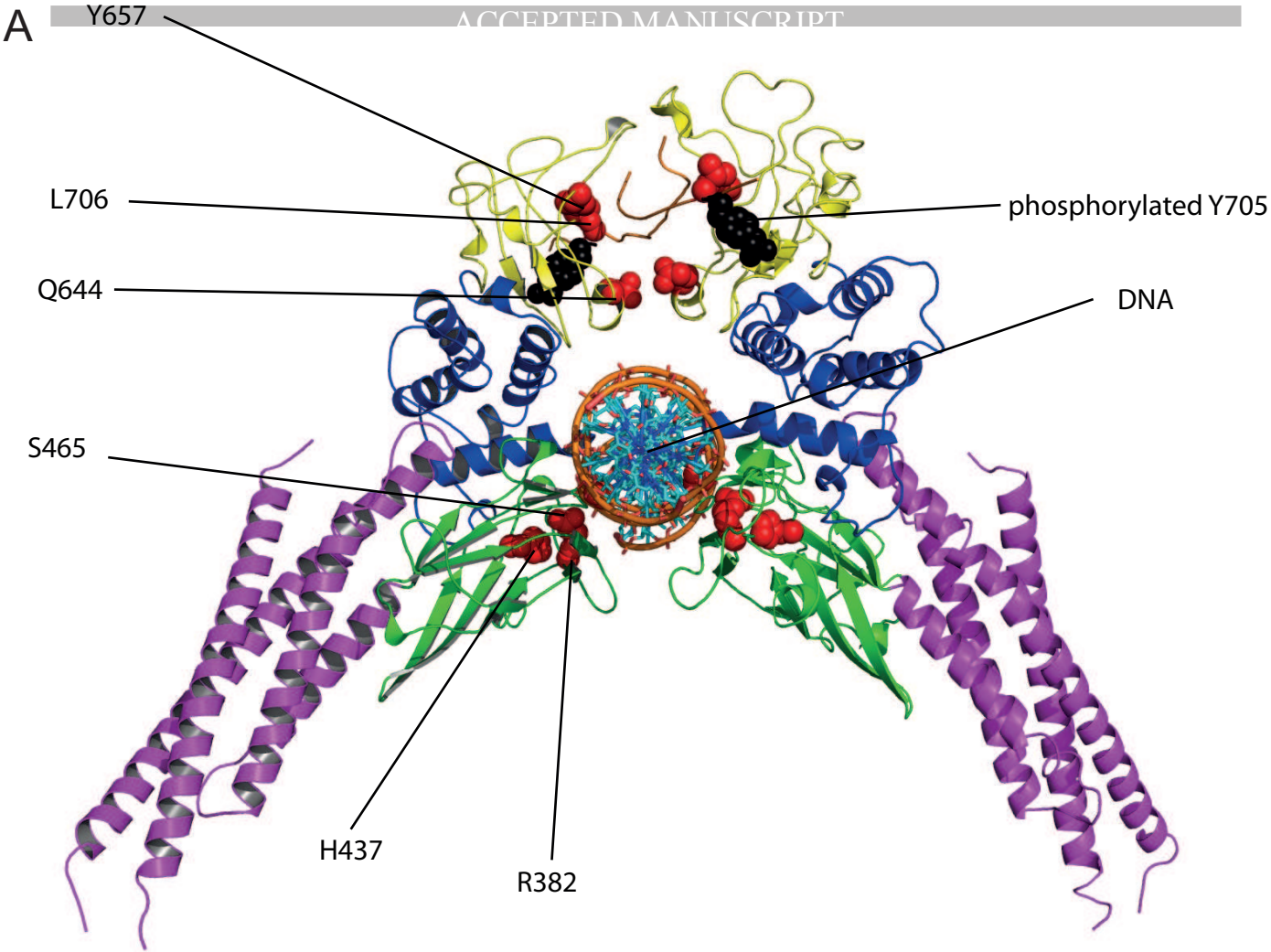


Figure S2

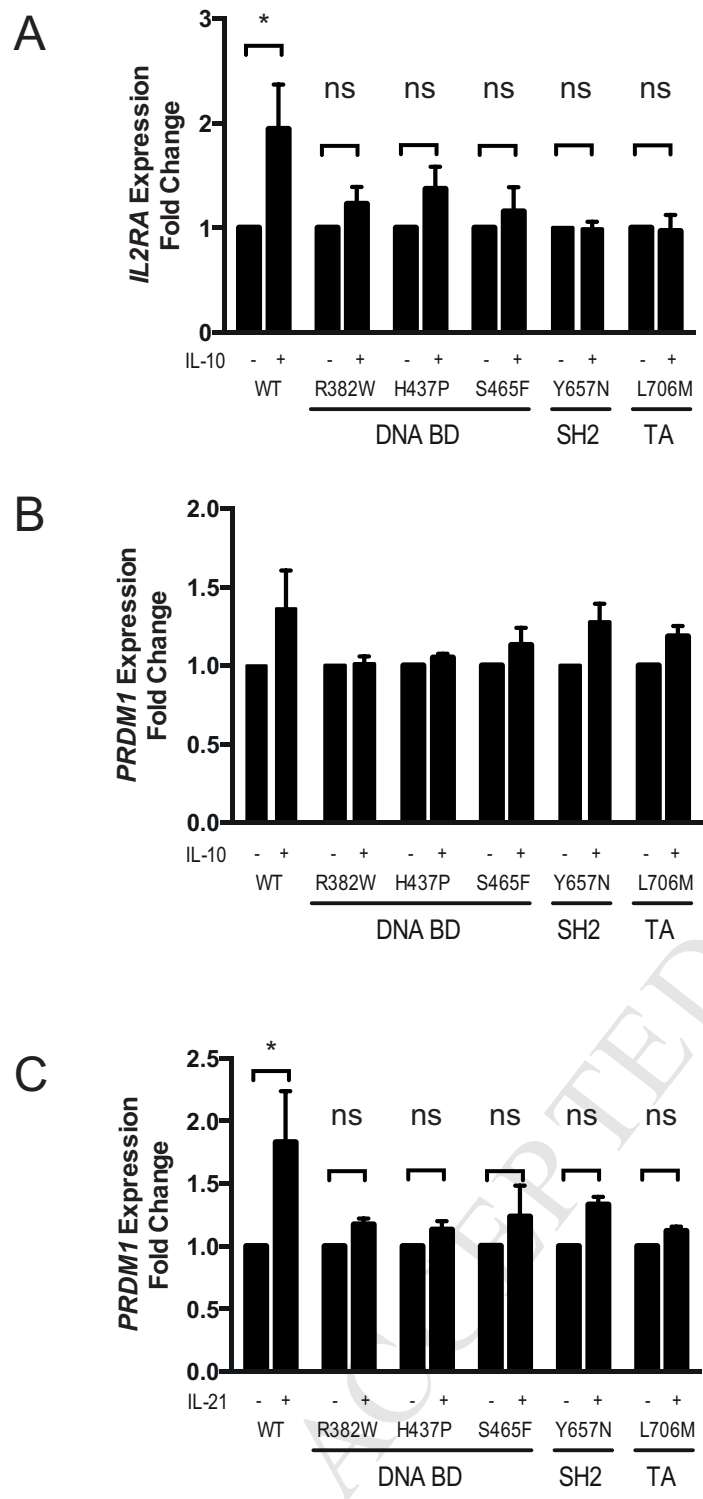


Figure S3

