

Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome

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Hyper-immunoglobulin E syndrome (HIES) is a compound primary immunodeficiency characterized by a highly elevated serum IgE, recurrent staphylococcal skin abscesses and cyst-forming pneumonia, with disproportionately milder inflammatory responses, referred to as cold abscesses, and skeletal abnormalities¹. Although some cases of familial HIES with autosomal dominant or recessive inheritance have been reported, most cases of HIES are sporadic, and their pathogenesis has remained mysterious for a long time. Here we show that dominant-negative mutations in the human signal transducer and activator of transcription 3 (STAT3) gene result in the classical multisystem HIES. We found that eight out of fifteen unrelated non-familial HIES patients had heterozygous STAT3 mutations, but their parents and siblings did not have the mutant STAT3 alleles, suggesting that these were *de novo* mutations. Five different mutations were found, all of which were located in the STAT3 DNA-binding domain. The patients' peripheral blood cells showed defective responses to cytokines, including interleukin (IL)-6 and IL-10, and the DNA-binding ability of STAT3 in these cells was greatly diminished. All five mutants were non-functional by themselves and showed dominant-negative effects when co-expressed with wild-type STAT3. These results highlight the multiple roles played by STAT3 in humans, and underline the critical involvement of multiple cytokine pathways in the pathogenesis of HIES.

Elevated serum IgE is a hallmark of many allergic disorders². Curiously enough, the hyper-IgE state is also observed in some primary immunodeficiency disorders, such as HIES, Wiskott-Aldrich syndrome, Omenn syndrome and Comèl-Netherton syndrome³. HIES (OMIM number 243700) was first reported in 1966 as Job's syndrome (OMIM number 147060)^{4,5}, but its underlying cause is still unknown, unlike the other three syndromes. In most cases of HIES, the clinical manifestations extend over multiple systems in the body, including the immune system, skeletal/dental system and soft tissue⁶. In contrast, the abnormalities in familial autosomal recessive (AR)-HIES patients seem to be confined to the immune system⁷. We previously identified a homozygous mutation of the tyrosine kinase 2 (TYK2) gene in a patient who showed AR-HIES and susceptibility to intracellular bacterial infections⁸. TYK2 is a non-receptor tyrosine kinase belonging to the JAK family^{9,10}. The patient's cells expressed no detectable TYK2 protein and displayed defects in multiple cytokine signals, including the signalling pathways for IL-6, IL-10, IL-12, IL-23 and type I IFN. The cytokine signals were successfully restored by introducing the intact TYK2 gene into the patient's cells. These multiple defects probably account for the patient's complex clinical manifestations⁸. The identification of a TYK2 deficiency in this HIES patient indicated to us that, besides TYK2, one or more molecules shared by multiple cytokine signalling pathways might also cause HIES.

To explore this possibility, we first examined the responses to IL-6, IL-10, IL-12 and IFN α of peripheral blood cells from two patients

(patient 1 and patient 2) who showed characteristics of multisystem HIES, including skeletal/dental abnormalities, skin abscesses, cyst-forming pneumonia and highly elevated serum IgE (Supplementary Table 1). The patients' B cells secreted IgM normally when stimulated with Epstein-Barr virus (EBV) infection (Fig. 1a). However, additional stimulation with IL-6 induced no significant increase in IgM secretion in the patients' B cells, unlike in the control B cells (Fig. 1a). Moreover, the suppression of lipopolysaccharide-induced production of TNF α by IL-10 deteriorated in the patients' macrophages (Fig. 1b). Thus, both the IL-6 and IL-10 pathways were defective in these HIES patients, as in the TYK2-deficient patient (Fig. 1a and 1b). In contrast, neither IL-12 nor IFN α signalling was impaired in the HIES patients, unlike in the TYK2-deficient patient (Fig. 1c and 1d). The HIES patients' T cells produced IFN γ normally in response to IL-12 (Fig. 1c), and their peripheral blood mononuclear cells (PBMCs) showed normal upregulation of transcripts for two IFN-inducible genes, *NMI* and *MX1* (also known as *MxA*), in response to IFN α (Fig. 1d). These observations indicated a possible abnormality in one or more molecules that was shared by the IL-6 and IL-10 signals but not essential for the IL-12 and IFN α pathways.

An array of cytokine signals is transduced by different combinations of JAK family kinases and STATs^{9,10}. When cytokines bind to their receptors, receptor-associated JAKs are activated to phosphorylate STATs, which in turn dimerize and translocate to the nucleus, where they activate target genes. In a survey of possible abnormalities in this signal cascade, we identified heterozygous mutations in the

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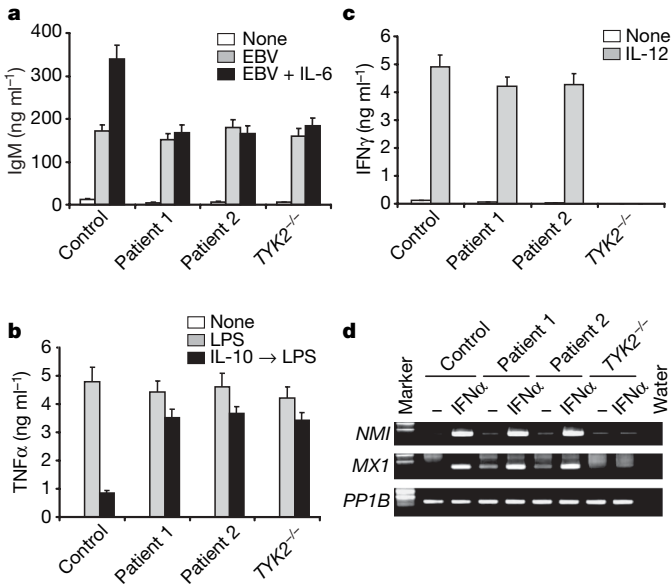


Figure 1 | Impaired responses to IL-6 and IL-10 in HIES patients' cells. **a**, IgM levels in culture medium of PBMCs from a control subject, two HIES patients (patient 1 and patient 2), and the TYK2-deficient patient, cultured for 7 days without stimulation, with EBV alone or with EBV and IL-6. **b**, TNFα levels in culture medium of macrophages from the same subjects, cultured without or with lipopolysaccharide (LPS) stimulation for 48 h, or with IL-10 treatment for 24 h before lipopolysaccharide stimulation. **c**, IFNγ in culture medium of CD4⁺ T cells from the same subjects, cultured for 24 h without or with IL-12. Error bars show standard deviations (**a–c**). **d**, NMI, MX1 and cyclophilin B (PP1B) transcript levels in PBMCs from the same subjects were unstimulated or stimulated with IFNα for 2 h.

STAT3 DNA-binding domain in both patients: a single amino acid deletion (ΔV463) in patient 1 and a mis-sense mutation (R382W) in patient 2 (Fig. 2). Because STAT3 is shown to be activated in response to a wide variety of cytokines, growth factors, and hormones^{11,12}, we thought that its mutation could well account for the patients' complex clinical manifestations extending over multiple systems. Further analysis of the STAT3 complementary DNA sequences in thirteen more unrelated patients with non-familial HIES identified heterozygous mutations in six of these patients (patients 3–8; see Supplementary Table 1 for clinical summary), and all of these mutations were located in the DNA-binding domain of STAT3: ΔV463 in

patient 3 and patient 8, like in patient 1; R382Q in patient 4; H437Y in patient 5; T389I in patient 6; R382W in patient 7, like in patient 2 (Fig. 2). The five different STAT3 mutations found in the eight patients were all confirmed by sequencing their genomic DNA, and no sequence alterations were detected in any other parts of STAT3, TYK2 or JAK1. The DNA-binding domain of STAT3 is highly conserved among different species in its amino acid sequence, and the alterations identified in the patients' STAT3 gene were statistically highly significant in population genetics ($P < 10^{-15}$, Fisher's exact probability test), as judged by the fact that such alterations were not found in 1,000 unrelated healthy individuals analysed, including at least 100 controls from the patients' ethnic group. STAT3 is located on human chromosome 17q21, but not 4q, which was reported to contain a disease locus for familial AD-HIES¹³. None of the eight HIES patients in the present study had a known family history of HIES, and no mutation was detected in the STAT3 cDNAs from all the parents and seven siblings of the patients, even though an analysis of multiple polymorphic markers confirmed the biological parent-child relationship (data not shown). Therefore, the mutations are likely to have occurred *de novo* in the HIES patients.

We next evaluated the biological significance of the STAT3 mutations. The STAT3 protein levels were comparable in all the EBV-transformed B-cell lines established from patients 1–6 and a control subject, and the extent of the tyrosine phosphorylation on STAT3 induced by IFNα stimulation was also comparable (Fig. 3a). Furthermore, all the mutant proteins formed a complex with wild-type STAT3 as efficiently as did wild-type STAT3, as judged by the co-immunoprecipitation of wild-type and mutant STAT3 proteins co-expressed in COS7 cells (Fig. 3b). However, nuclear extracts isolated from the patients' cells stimulated with IFNα contained much lower amounts of active STAT3 that could bind to target DNA compared with nuclear extracts from the control cells, whereas the DNA-binding activity of STAT1 was intact in the patients' cells (Fig. 3c). This finding was consistent with a previous report showing that changing codons 461 to 463 in STAT3 from Val-Val-Val to Ala-Ala-Ala resulted in the impairment of STAT3's DNA-binding activity¹⁴. Thus, the genetic mutations identified in the HIES patients seemed to result in the impairment of the DNA-binding activity of STAT3 and most likely that of heterodimers between mutant and wild-type STAT3 molecules. A similar impairment of DNA-binding activity in STAT1 protein was recently reported in cells isolated from patients carrying a heterozygous mutation in the DNA-binding domain of STAT1 (ref. 15).

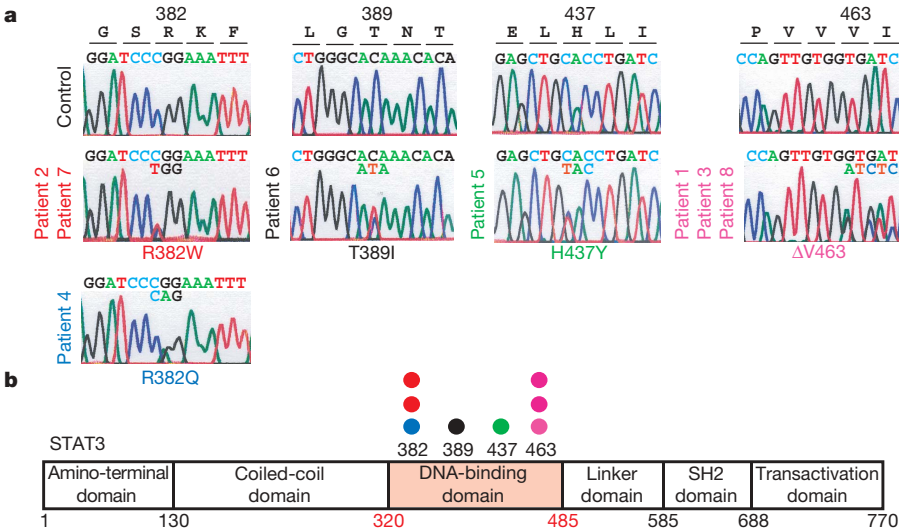


Figure 2 | Heterozygous mutations in the DNA-binding domain of STAT3 from eight HIES patients. **a**, Electropherograms showing partial STAT3 cDNA sequences from a control subject and the eight HIES patients. **b**, The

structure of STAT3 is shown schematically, and the positions of the mutations identified in the eight patients are indicated.

When wild-type STAT3 was exogenously expressed—together with a luciferase reporter gene containing STAT3-responsive elements—in human HeLa cells in which the endogenous STAT3 was knocked down, fivefold upregulation of luciferase activity in response to IFN α was detected (Fig. 4a). In contrast, none of the STAT3 mutants conferred any significant increase of luciferase activity on the HeLa cells in response to IFN α , demonstrating a loss of function of the STAT3 mutants. To explore the possibility that the STAT3 mutant proteins function as dominant-negative, wild-type STAT3 or the individual mutants were exogenously expressed in IL-6-responsive HepG2 cells and IL-10-responsive MC/9 cells (Fig. 4b and 4c). In HepG2 cells transfected with empty vector together with the reporter luciferase gene, the luciferase activity was upregulated as much as 3.5 times its basal level in response to IL-6 stimulation. The IL-6-induced upregulation of luciferase activity was augmented in HepG2 cells transfected with wild-type STAT3 by up to 5.5 times, whereas it was severely impaired in cells transfected with any of the mutant STAT3s, showing at most a 2-fold increase (Fig. 4b). Moreover, the IL-10-induced downregulation of surface KIT (C-Kit) expression was severely impaired in MC/9 cells transfected with any of the mutant STAT3s, unlike those with empty

vector or wild-type STAT3 (Fig. 4c). Thus, all the STAT3 mutants identified in the HIES patients displayed dominant-negative effects when co-expressed with wild-type STAT3.

The DNA-binding activity of STAT3 in the IFN α -stimulated patients' cells was not totally abrogated, although it was only approximately one-fourth that of control cells (Fig. 3b). This residual STAT3 activity might have rescued the patients from early embryonic death, which is observed in STAT3-deficient mice¹⁶. In contrast, its diminished activity might have an impact on the development and functions of multiple organ systems, leading to compound clinical manifestations of HIES, given STAT3's role in the signalling pathways of a variety of soluble factors, including the IL-6-family cytokines (IL-6, IL-11, IL-27, IL-31, LIF, OSM, CNTF and cardiotrophin-1), the IFN-family cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IFN α / β and IFN γ), the IL-2-family cytokines (IL-2, IL-7, IL-9, IL-15 and IL-21), IL-5, IL-23, CSF3/G-CSF, EGF, CSF1 and leptin^{12,17,18}.

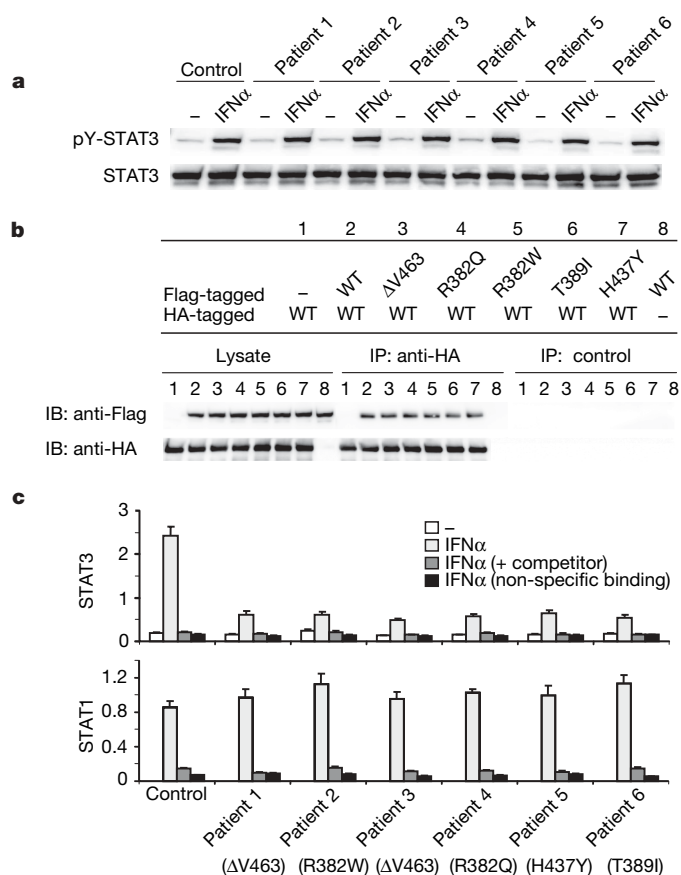


Figure 3 | Diminished DNA-binding activity of STAT3 in the HIES patients' cells. **a**, Total and tyrosine-phosphorylated (pY-) STAT3 proteins detected by immunoblotting. EBV-transformed B-cell lines (EBV-LCL) established from a control subject and the HIES patients were unstimulated or stimulated with IFN α for 15 min. **b**, Association of Flag- or HA-tagged wild-type (WT) and mutant STAT3s co-expressed in COS7 fibroblast cells was examined by immunoprecipitation (IP) with anti-HA or control antibody followed by immunoblotting (IB) with anti-Flag or HA antibody. **c**, DNA-binding activity of STAT3 and STAT1 in nuclear extracts prepared from EBV-LCLs from a healthy control and the HIES patients that were unstimulated or stimulated with IFN α for 15 min. Dark grey bars indicate the DNA-binding activity in the presence of competitor oligonucleotides, and black bars indicate non-specific binding to irrelevant oligonucleotides. Error bars are standard deviations.

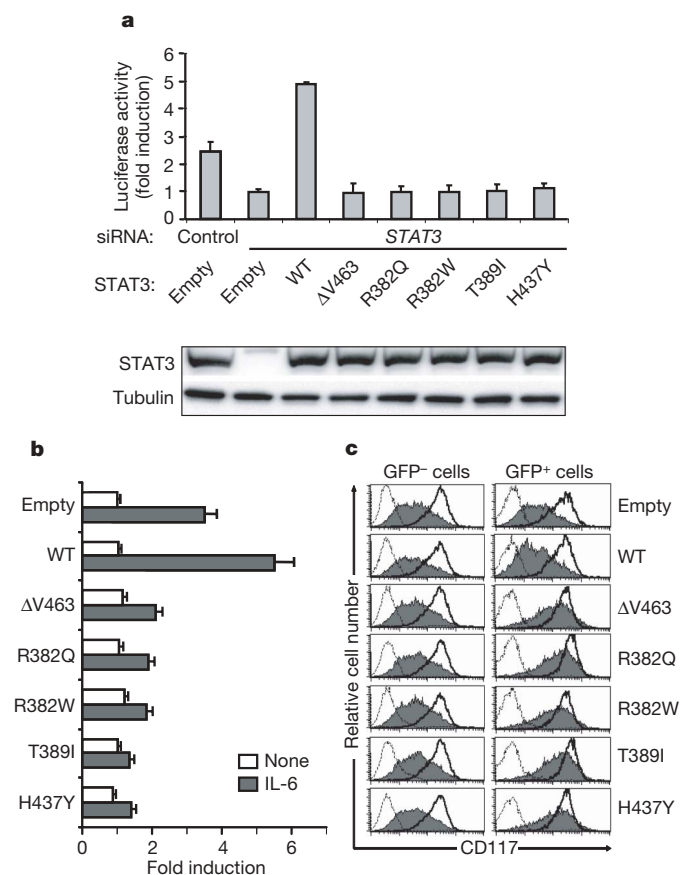


Figure 4 | Loss-of-function and dominant-negative effect of the STAT3 mutants in cytokine signals. **a**, Wild-type or individual mutants of STAT3 were exogenously expressed together with a luciferase reporter gene containing STAT3-responsive elements in human HeLa cells in which endogenous STAT3 was knocked-down by transfecting with two sets of siRNA oligonucleotides. The HeLa cells were then left unstimulated or stimulated with 10 ng ml⁻¹ of IFN α for 5 h. The relative luciferase activity in the cell lysates of IFN α -stimulated versus unstimulated cells is shown (error bars show standard deviations). Expression of endogenous and exogenous STAT3 proteins detected by immunoblotting is shown in the lower panel. **b**, The relative luciferase activity in the cell lysates of unstimulated or IL-6-stimulated HepG2 cells that were transfected with the luciferase reporter construct plus an empty vector or a vector containing the wild-type (WT) or one of each mutant STAT3 sequence. Error bars show standard deviations. **c**, The CD117 expression level on untreated (thin line histograms) or IL-10-treated (shaded histograms) MC/9 cells that were infected with retroviral vectors containing WT or one of the mutant STAT3 sequences. Data are shown for GFP⁺ infected and GFP⁻ uninfected cells, and thin dotted histograms indicate staining with a control antibody.

It has been shown that STAT3 has important roles in the differentiation of both osteoblasts and osteoclasts *in vitro*¹⁹, and mice deficient for STAT3 in osteoblasts show an osteoporotic phenotype²⁰. When osteoclasts were generated from peripheral blood monocytes in culture with CSF1/M-CSF and TNFSF11/RANKL, those from the HIES patients with the STAT3 mutations showed higher bone-resorption activity compared to those from control subjects (Supplementary Fig. 1). This may reflect the skeletal/dental abnormalities observed in HIES patients. Another remarkable feature of HIES is that patients are often afebrile and feel well¹, despite serious pneumonia or dermal pathology⁴. Indeed, acute-phase responses, such as an increase in serum C-reactive protein during severe infections, were diminished in our patients. STAT3 was originally identified as a protein binding to the IL-6-responsive element in the genes encoding hepatic acute-phase proteins^{21,22}, and the liver-specific inactivation of STAT3 leads to an impaired acute-phase response in mice²³. Thus, the apparent lack of classical inflammatory responses in HIES patients could be attributed to defective signalling of pro-inflammatory cytokines, including IL-6.

Enhanced IgE production in the patients may reflect dysregulated immune responses owing to the impaired response to IL-10, a critical negative regulator²⁴, even though the exact mechanism of hyper IgE remains to be determined, as in the case of other disorders such as Wiskott-Aldrich syndrome. HIES patients often suffer from severe staphylococcal infection in the skin and lung. STAT3 plays a critical part in T_H17 development²⁵, and IL-17 produced by T_H17 cells is protective in the host defence against extracellular bacteria²⁶. IL-22 stimulates cells in the skin and respiratory systems to produce β -defensins through STAT3 activation²⁷. Thus, the susceptibility to bacterial infection could be attributed, at least in part, to the defects in T_H17 development and IL-22 signalling. Among the 15 sporadic HIES patients investigated in this study, no apparent difference was observed in clinical phenotypes and severity between those with the STAT3 mutations and those without the mutations, indicating that other HIES aetiology might be functionally linked to STAT3.

In summary, the present study identified a human deficiency in STAT3 as a major cause of multisystem HIES. This study highlights the multiple and critical roles of STAT3 in humans. The identification of these STAT3 mutations as causative for HIES, in addition to the previous finding of a causative mutation in TYK2 (ref. 8), underlines the critical involvement of a variety of cytokine signals in the pathogenesis of HIES. The diagnosis of HIES early in life is often hampered by a paucity of specific clinical features. Our discovery of STAT3 as a major causative gene of this disease will facilitate earlier and definitive diagnosis, leading to the prevention of serious complications by prompting the start of prophylactic antibiotic treatment early in life.

METHODS SUMMARY

Patients. This study was approved by the institutional review board at the Tokyo Medical and Dental University; written informed consent was obtained from all the individuals studied. The clinical characteristics of the eight HIES patients investigated in this study are summarized in Supplementary Table 1, and all the patients display definitive phenotypes of multisystem HIES (score ≥ 40).

Stimulation of cells with cytokines, and measurement of cytokines and IgM production. Cells were stimulated for the indicated time in culture with cytokines as described previously⁸. The concentration of IFN γ and TNF α in the culture supernatants was determined by ELISA (BD-PharMingen), according to the manufacturer's instructions. The amount of IgM secretion from EBV-infected B cells was determined as previously described²⁸.

RT-PCR and direct sequence analysis. Extraction of total RNA, cDNA synthesis, PCR, semiquantitative RT-PCR, and sequencing were performed as previously described²⁹.

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation were performed as described previously⁸.

Enzyme-linked DNA-protein interaction assay. Binding of STAT3 and STAT1 to their target DNA was measured using the Mercury TransFactor kit (Clontech Laboratories) according to the manufacturer's protocol.

Retroviral infections. Retroviral infections were done as described previously⁸.

Flow cytometric analysis. The surface immunophenotype was analysed as described³⁰.

STAT3 knock-down. Transfection of short interfering RNA (siRNA) oligonucleotides (5'-ccugaagagucgauguucuu-3' and 5'-gcaguucucagagcag-guauuu-3') was performed as described previously⁸. Forty hours after transfection, the cells were treated with IFN α for 5 h. In the experiment shown in Fig. 4a, nucleotide sequences of wild-type and mutant STAT3 cDNAs were modified so that they were insensitive to STAT3 siRNA, but they still encoded the original amino acid sequences of STAT3.

Luciferase reporter assay. The reporter construct of STAT3-responsive elements linked to a luciferase reporter gene was transfected with wild-type or mutant STAT3. Forty hours after the transfection, the cells were stimulated with 100 ng ml⁻¹ IL-6 for 5 h. Luciferase activity was measured with a dual-luciferase assay system according to the manufacturer's protocol (Promega).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Patients. An immunological work-up revealed high serum IgE in all the patients and eosinophilia in five of them. All other laboratory data examined were within the normal range, including the lymphocyte subpopulations, their proliferative responses to mitogens, the levels and subclasses of serum immunoglobulins, the oxidative burst of granulocytes, and the number and size of platelets.

Antibodies and cytokines. Antibodies against STAT3, tyrosine-phosphorylated STAT3, Flag and HA, and HRP-conjugated rabbit anti-mouse and goat anti-rabbit antibodies were purchased from Cell Signaling. The CD117 monoclonal antibody was from BD-PharMingen, and the CD3 monoclonal antibody (OKT3) was from Janssen Pharmaceutical. Recombinant human IL-6, IL-10, IL-12, IFN α , and GM-CSF were purchased from Peprotech, recombinant human IL-2 from Shionogi, and lipopolysaccharide (055:B5) from Sigma-Aldrich.

Isolation and culture of T cells and macrophages from PBMCs. Isolation and cell culture of T cells and macrophages were performed as described previously⁸. All the experiments were performed at least three times with three different controls.

Stimulation of cells with cytokines, and measurement of cytokines and IgM production. Cells were stimulated for the indicated time in culture with IL-6 (100 ng ml⁻¹), IL-10 (100 ng ml⁻¹), IL-12 (10 ng ml⁻¹), or IFN α (5 ng ml⁻¹) as described previously⁸.

RT-PCR and direct sequence analysis. Sequencing was performed with an ABI Prism dRhodamine Terminator kit and analysed with an ABI Prism 310 DNA Sequencer (Perkin-Elmer Applied Biosystems). At least two independent PCR products were sequenced.

Enzyme-linked DNA-protein interaction assay. Thirty micrograms of nuclear extracts were incubated in a 96-well microplate precoated with oligonucleotides encoding the consensus binding sequence for STAT1 or that for STAT3. Bound STAT3 or STAT1 was detected with specific antibodies plus an HRP-conjugated secondary antibody.

Retroviral infections. Retroviral infections were done with the retroviral vector pMX-IRES-GFP (a gift from T. Kitamura) carrying the wild-type or one of each mutant STAT3 sequence as described previously⁸.

STAT3 knock-down. Transfection of siRNA oligonucleotides was performed by using Lipofectamine-RNAiMAX reagent (Invitrogen). Forty hours after transfection, the cells were treated with IFN α (10 ng ml⁻¹) for 5 h.

Luciferase reporter assay. The reporter construct contained 4 repeated STAT3-responsive elements linked to a luciferase reporter gene. HeLa cells or HepG2 cells were transfected with the pcDNA3 vector bearing wild-type or mutant STAT3, the reporter construct, and an expression vector for *Renilla* luciferase driven by the CMV reporter, with FuGENE6 (Roche). The relative luciferase activity was determined by normalizing the values against the *Renilla* luciferase signal.

SUPPLEMENTARY INFORMATION

Figure S1

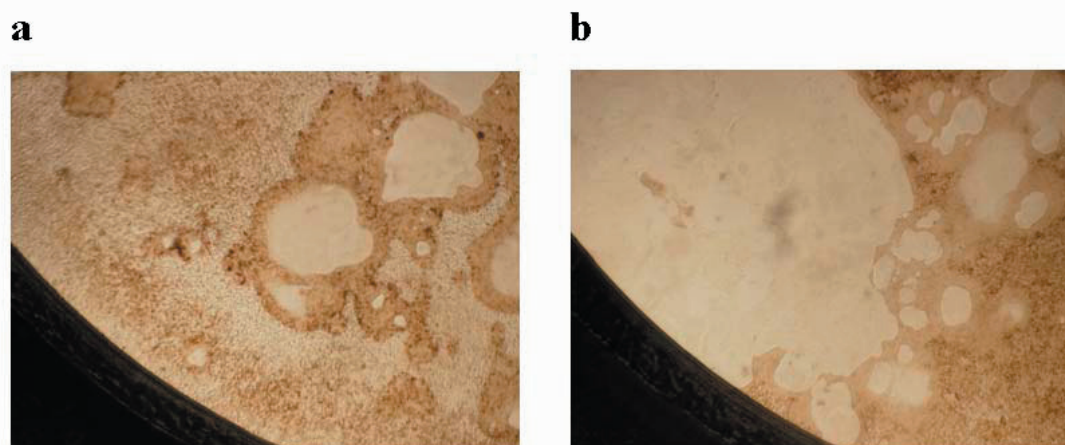


Figure S1: CD14⁺ monocytes enriched from PBMCs of the HIES patients or control subjects were cultured in the presence of receptor activator of NF- κ B ligand (RANKL; 40 ng/ml) and macrophage colony-stimulating factor (M-CSF; 25 ng/ml) for 14 days on a hydroxy appatite-coated disc (Osteologic; BD Biosciences) to generate osteoclasts. After osteoclasts were removed from the

disc as described in the manufacturer's protocol, resorption lacunae (pale areas on the disc) formed by osteoclasts were evaluated by light microscopic examination. Representative photographs from a control subject (a) and the HIES patient 1 (b) are shown.

Table S1: Clinical findings and scores of 8 HIES patients investigated in this study

HIES patients	Pt1	Pt2	Pt3	Pt4	Pt5	Pt6	Pt7	Pt8	Tyk2 ^{-/-} Pt
Age	21	9	19	10	13	7	7	6	23
Sex	F	F	M	M	F	F	M	M	M
Clinical findings common to all types of HIES									
Highest serum IgE level	10	10	10	10	10	10	10	10	10
Highest eosinophil count	0	6	6	0	6	6	6	6	3
Skin abscesses	2	8	8	8	8	8	2	8	8
Pneumonia (episodes/lifetime)	8	8	0	8	8	8	8	6	8
Newborn rash	4	4	4	4	4	4	4	4	4
Eczema	2	4	4	4	4	2	1	4	4
Upper respiratory infection/yr	0	2	0	0	2	1	1	2	2
Candidiasis	0	1	4	0	4	0	2	2	1
Other serious infection	0	0	4	0	0	0	0	4	4
Fatal infection	0	0	0	4	0	0	0	0	4
Lymphoma	0	0	0	0	0	0	0	0	0
Subtotal score	26	43	40	38	46	39	34	46	48
Findings characteristic to multisystem HIES									
Parenchymal lung anomalies	8	8	0	0	0	8	8	8	0
Retained primary teeth	8	8	0	0	2	0	0	0	0
Scoliosis	4	0	0	0	0	0	2	0	0
Fractures with minor trauma	0	0	0	0	0	0	4	4	0
Characteristic face	5	2	5	2	2	2	2	5	0
Midline anomaly	0	0	0	0	0	0	0	0	0
Hyperextensibility	4	4	0	0	0	0	4	4	0
Increased nasal width	3	1	1	1	1	1	1	1	0
High palate	0	0	2	0	0	0	2	0	0
Subtotal score	32	23	8	3	5	11	23	22	0
Total score	58	66	48	41	51	50	57	68	48

Each clinical finding is scored according to the HIES scoring system (Grimbacher et al. *Am. J. Hum. Genet.* 65: 735-744, 1999).