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BRIEF REPORT

Cytomegalovirus Infection Mimicking Juvenile Myelomonocytic Leukemia Showing Hypersensitivity to Granulocyte–Macrophage Colony Stimulating Factor

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We describe an infant with cytomegalovirus (CMV) infection presenting as transient myeloproliferation resembling juvenile myelomonocytic leukemia (JMML). The patient fulfilled the international diagnostic criteria of JMML, including hypersensitivity to granulocyte–macrophage colony-stimulating factor (GM-CSF). Viral studies using serologic assays and polymerase chain reaction (PCR) were positive for CMV. Clinical symptoms disappeared and laboratory values returned to normal without specific treatment within 1 year. Follow-up showing a decrease in viral titers suggested CMV infection as an etiologic factor for the development of myeloproliferative features. We conclude that the CMV infection transiently induced abnormal myelopoiesis in this infant. Pediatr Blood Cancer 2009;53:1324–1326. © 2009 Wiley-Liss, Inc.

Key words: cytomegalovirus; juvenile myelomonocytic leukemia; GM-CSF hypersensitivity

INTRODUCTION

The majority of prenatal and postnatal cytomegalovirus (CMV) infections are asymptomatic in newborn periods; however, clinical features of symptomatic CMV infection often overlap other hematologic diseases. Juvenile myelomonocytic leukemia (JMML) is a myeloproliferative/myelodysplastic disorder that primarily affects children younger than 5 years of age. However, there are several reports describing difficulties in discriminating between JMML and infectious diseases [1,2]. The diagnosis of JMML is based on the presence of defined diagnostic criteria including a characteristic hypersensitivity of myeloid progenitors to granulocyte–macrophage colony-stimulating factor (GM-CSF) [2]. We present a case with CMV infection showing GM-CSF hypersensitivity mimicking JMML.

CASE REPORT

A 2-month-old Japanese female with a 1 month history of failure to thrive and repeated infections was referred to our hospital. Physical examinations revealed hepatosplenomegaly with the liver descended 5 cm below the right costal margin and the spleen 4 cm below the left costal margin. Laboratory data showed a white blood cell count of 12.9×10^9/L; hemoglobin, 10.4 g/dl; and platelets, 265×10^9/L. The differential count showed elevated monocytes and immature granulocytes (23% neutrophils, 49% lymphocytes, 4% eosinophils, 14% monocytes, 7% myelocytes, and 3% metamyelocytes). Hemoglobin F was normal at 30.8% (normal range for age: 25–60%). Serologic tests and/or polymerase chain reaction (PCR) analysis for Epstein–Barr virus (EBV), human herpesvirus (HHV)-6, and parvovirus B19 were negative. IgM titer for CMV by enzyme linked immunosorbent assay was positive and the existence of CMV infection was further confirmed by PCR.

The bone marrow was hypercellular with a myeloid/erythroid ratio of 4.6:1. Blasts and promyelocytes comprised 0.4% of nucleated cells. No myelodysplasia was seen in the bone marrow. Karyotyping of marrow cells revealed 46XX, with no Philadelphia chromosome or monosomy 7. To differentiate JMML from CMV infection, in vitro culture assays of bone marrow and peripheral blood were examined [3]. The results showed spontaneous proliferation of predominantly monocytic/macrophage colonies. In vitro assays showed the patient's cells were hypersensitive to GM-CSF (Table I).

Though the patient fulfilled the criteria of JMML [2], no specific treatment was required because no sign of progressive disease was seen. The patient was closely monitored and her clinical course was unremarkable with gradual resolution of hepatosplenomegaly and blood counts. Peripheral blood monocyte count dropped below

TABLE I. Spontaneous CFU-GM Formation and GM-CSF Dose–Response Analysis From Patient Samples

GM-CSF (ng/ml)	Peripheral blood (normal range)	Bone marrow (normal range)
0	73 (0–8)	78 (0–3)
0.01		103 (1–6)
0.1		118 (3–6)
1		129 (5–14)
10		126 (23–46)

The colony assays were performed as previously described [3]. The depletion of monocytes was employed for bone marrow cells. 1×10^5 cells were used for peripheral blood whereas 2×10^4 cells were cultured for bone marrow cells. CFU-GM, colony forming unit-granulocyte and macrophage; GM-CSF, granulocyte–macrophage colony-stimulating factor.

Additional Supporting Information may be found in the online version of this article.

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MAJOR ARTICLE

Risk Parameters of Fulminant Acute Respiratory Distress Syndrome and Avian Influenza (H5N1) Infection in Vietnamese Children

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A clinical picture of patients with acute respiratory distress syndrome (ARDS) induced by highly pathogenic avian influenza A (H5N1) has been reported. We reviewed 37 sets of clinical data for pediatric patients with ARDS at the National Hospital of Pediatrics (Hanoi, Vietnam); 12 patients with H5N1-positive and 25 with H5N1-negative ARDS were enrolled. The H5N1-negative patients had a clinical picture and mortality rate similar to that for the pediatric ARDS patients. However, the H5N1-positive patients had ARDS with normal ventilation capacity at the time of hospital admission, then rapidly proceeded to severe respiratory failure. The survival probability and days until final outcome in groups of H5N1-positive (n = 12) vs. H5N1-negative (n = 25) patients were 17% versus 52% and 12.3 ± 5.7 days (median, 11 days) versus 21.5 ± 13.8 days (median, 22 days), respectively. Our observations clarified the clinical picture of H5N1-induced fulminant ARDS and also confirmed that relatively older age (~6 years of age), high fever at onset, and leukopenia and/or thrombocytopenia at the time of hospital admission are risk parameters for H5N1-induced fulminant ARDS.

Highly pathogenic avian influenza A (H5N1) came to the attention of the international scientific community for the first time in 1997 [1, 2]. The current global spread of human infection by this subtype started in 2003 in Hong Kong [2, 3], during the global outbreak of severe acute respiratory syndrome [4, 5]. Vietnam reported the first human case of H5N1 infection in January 2004 [6] and a suspected human-to-human transmission family cluster in the following months [7]. Since then, many clinical case reports have been reported from several countries, such as Thailand, Indonesia, and Vietnam [8–14]. However, it is still difficult to detect most infection at first examination without a clear history of patient contact with sick poultry.

The fatality rate associated with pediatric acute respiratory distress syndrome (ARDS) has decreased during recent decades because of advances in medical treatment, especially respiratory management as a lung-protective therapy [15]. However, the majority of patients with H5N1 subtype influenza virus infection experienced or presented ARDS during their clinical courses, often followed by a serious outcome. The histopathology of these cases demonstrated diffuse alveolar damage in the lung, which also suggests ARDS as a clinical condition of the respiratory system [16–18]. Because of the significant possibility that H5N1 subtype influenza will the source of the next pandemic influenza strain [19, 20], the pathophysiology of the clinical course of H5N1

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Hyper-IgE syndrome

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Hyper-IgE syndrome (HIES) is a complex primary immunodeficiency characterized by atopic dermatitis associated with extremely high serum IgE levels and susceptibility to infections with extracellular bacteria. Nonimmunological abnormalities, including a distinctive facial appearance, fracture following minor trauma, scoliosis, hyperextensive joints, and the retention of deciduous teeth are also observed in most patients. Recent studies have demonstrated that *dominant-negative* mutations in the signal transducer and activator of transcription 3 (*STAT3*) gene result in the classical multisystem form of HIES, whereas a null mutation in the tyrosine kinase 2 (*TYK2*) gene causes an autosomal recessive HIES associated with viral and mycobacterial infections. In both patients, signal transduction for multiple cytokines, including IL-6 and IL-23, was defective, resulting in impaired T_H17 function. These findings suggest that the defect in cytokine signaling constitutes the molecular basis for the immunological and nonimmunological abnormalities observed in HIES.

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Introduction

Hyper-IgE syndrome (HIES) is a complex primary immunodeficiency disorder (PID) characterized by recurrent staphylococcal infections in the skin and lung, chronic eczema, and markedly high serum IgE concentrations [1–5]. Davis and Wedgwood first described this disease in 1966, in two girls suffering from recurrent 'cold' staphylococcal abscesses, pneumonia, and neonatal-onset eczematoid rash [6]. As this original report predated the identification of IgE [7], a high serum concentration of IgE level was not recognized in these patients. This syndrome was characterized further by Buckley *et al.* [8], who found that recurrent staphylococcal abscesses and chronic eczema were associated with exceptionally high serum concentrations of IgE. They also showed that serum concentrations of the other immunoglobulins (IgG, IgA, IgM, and IgD) and IgG subclasses were normal in the patients. The multisystem nature of HIES was established later, when manifestations of the disease were shown to extend beyond immune system abnormalities, to encompass skeletal and connective tissue abnormalities, such as scoliosis, osteoporosis, fracture following minor trauma, hyperextensive joints, and the retention of deciduous teeth [9]. In 2004, a novel form of HIES was identified in consanguineous families, suggesting the presence of autosomal recessive (AR) forms of HIES [10]. In 2006, a tyrosine kinase 2 (TYK2) deficiency was identified in an AR-HIES patient associated with susceptibility to intracellular bacterial and viral infections [11]. Finally, in 2007, *dominant-negative* mutations in the signal transducer and activator of transcription 3 (STAT3) gene were identified as a major molecular etiology of classical HIES [12••,13]. In this review, I will outline the clinical signs, molecular origins, and pathogenesis of HIES. I will also discuss several unanswered questions concerning the pathogenesis of HIES and potential directions for future research.

Clinical manifestations

Almost all patients with HIES suffer from recurrent staphylococcal infections, beginning in infancy and predominantly involving the skin and lungs [1–5,14]. This situation contrasts starkly with that in patients with chronic granulomatous disease (CGD), in which recurrent staphylococcal infections occur in a wide variety of organs, including the lung, lymph nodes, skin, liver, bone, gastrointestinal tract, kidney, and brain [15]. *Staphylococcus aureus* is the bacterium most frequently isolated from HIES patients, but *Streptococcus pneumoniae*, *Haemophilus influenzae*, and enteric Gram-negative bacteria are occasionally isolated from HIES patients, during episodes of infection. Fungal infections, including mucocutaneous candidiasis and pulmonary aspergillosis, are also common in HIES. Eczema usually begins during the neonatal period, before the onset of atopic dermatitis. Patients with HIES suffer from atopic dermatitis associated with extremely high serum IgE levels and eosinophilia, but are usually free from other allergic manifestations, such as allergic rhinitis, asthma, urticaria, and anaphylaxis.

In this review, HIES is classified into two categories, type 1 and type 2, regardless of its mode of inheritance. Patients with type 1 HIES have abnormalities in multiple systems of the body, including the skeletal and dental systems,

Table 1

A classification of HIES

HIES type	Inheritance	Discriminant clinical findings
Type 1	Sporadic (more than 90% of cases) Familial with autosomal dominant inheritance (rare)	Skeletal and connective tissue abnormalities (characteristic facial appearance, fracture following minor trauma, retention of deciduous teeth, scoliosis, hyperextensibility), pneumatocele
Type 2	Familial with autosomal recessive inheritance	Viral infections (herpes simplex virus, molluscum contagiosum) Central nervous system involvement Some mycobacterial infections Absence of pulmonary cysts Absence of skeletal abnormalities

whereas patients with type 2 HIES have abnormalities confined to the immune system (Table 1). Type 1 HIES is the most common form, and was the type presented by the cases reported by Davis and Buckley [6,8]. In this type of HIES, pneumonia is frequently followed by the formation of pneumatocele or pulmonary cysts, frequently complicated by infections with *Aspergillus* and multidrug-resistant *Pseudomonas aeruginosa* [16•]. Patients with type 2 HIES have no skeletal abnormalities, but suffer from recurrent viral infections, such as molluscum contagiosum and herpes simplex virus (HSV) infections [10]. No pneumatocele was found in patients with type 2 HIES. Most type 2 HIES patients have been found to have mild defects in signal transduction downstream from the T-cell receptor (TCR) complex.

Etiology of HIES

A null mutation in TYK2 results in a variant form of type 2 HIES

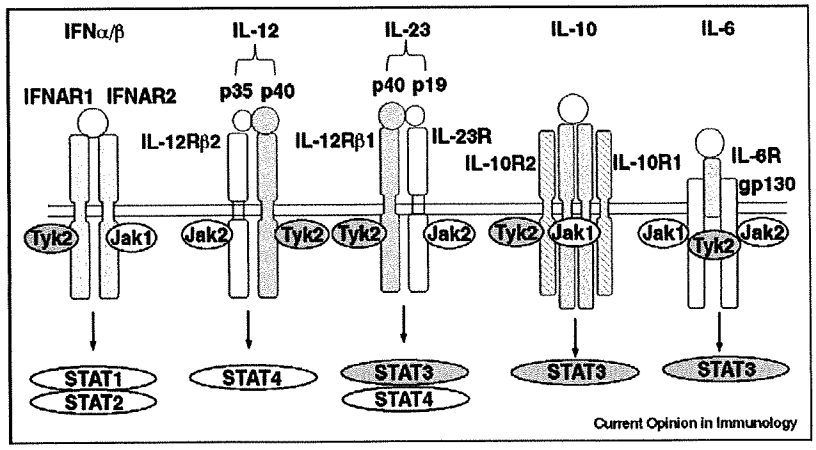
TYK2 deficiency was identified as a molecular cause of type 2 HIES based on the finding that a type 2 HIES patient was susceptible to intracellular bacterial infection and had a defect in signal transduction for IL-12 and IFNα [11]. Unlike other patients with type 2 HIES, this TYK2-deficient patient displayed intact TCR signaling. TYK2 is one of the founding members of the Janus kinase family (Jaks), transducing a signal downstream from a number of cytokines [17,16•,18–21]. The patient had a homozygous four-base pair deletion in the coding region of the *TYK2* gene, resulting in a premature stop codon and the absence of TYK2 protein. The parents of the patient were consanguineous and heterozygous for the 4-bp deletion. Both parents displayed normal immune responses, consistent with the AR inheritance of TYK2 deficiency. This disorder was named AR-HIES with mycobacterial and viral infections by the PID classification committee of the International Union of Immunological Societies (IUIS) [22].

Cells from the patient displayed severe defects in response to a number of cytokines, including type 1 IFN, IL-12, IL-23, IL-10, and IL-6 (Figure 1) [11]. The signal transduction initiated by these cytokines was restored by transduction with the wild-type *TYK2 in vitro*, suggesting that TYK2 deficiency was responsible for the clinical signs observed in the patients. The susceptibility of the patient to viral infections probably resulted from the defects in type 1 IFN signaling. IFNα-induced tyrosine phosphorylation of JAK1, STAT1, and STAT2 was abolished. Furthermore, no inhibition of HSV replication was observed after treatment of the patient's cells with a large dose of IFNα *in vitro*. Thus, TYK2 plays a nonredundant role in type 1 IFN signaling in humans, but not in mice [23,24]. Patients with *STAT1* mutations impairing both IFNγ and INFα/β signaling suffered from both mycobacterial disease and disseminated HSV-1 infection [25]. These data indicate that human type 1 IFN plays a crucial role in the immunological control of HSV. The absence of TYK2 resulted in defective IL-12 signaling, leading to impaired T_H1 differentiation and IFNγ production and susceptibility to intracellular bacterial infections. This is consistent with the observation that patients with IL-12B and IL-12RB1 deficiencies are susceptible to intracellular bacterial infections [26]. Impaired Th1 differentiation and accelerated Th2 differentiation probably resulted in atopic dermatitis, asthma, and high serum IgE levels in the TYK2-deficient patient.

A dominant-negative mutation in *STAT3* causes type 1 HIES

The identification of TYK2 deficiency in a type 2 HIES patient led us to investigate the responses to multiple cytokines in type 1 HIES patients. Signaling was found to be defective for IL-6 and IL-10, whereas signaling was intact for IL-12 and IFNα. In a survey of candidate genes, we identified heterozygous mutations affecting the DNA-binding domain of STAT3 in patients [12••]. None of the HIES patients had a family history of HIES, and no mutation was detected in the parents. These mutations therefore probably occurred *de novo* in the patients. *STAT3* mutations were subsequently identified in most of the patients with type1 HIES, including the patients described in the first report of HIES [13,27•,28,29]. These mutations were located in the DNA-binding domain in our eight patients, and were subsequently shown to affect the Src-homology 2 (SH2) domain, the linker domain, and the transactivation domain of STAT3 [30].

Figure 1



Cytokine receptors and the associated intracellular Jaks (Jak1, Jak2, and Tyk2) and STATs.

STAT3 is a transcription factor that binds to the STAT3-responsive elements in the promoters of various genes, including those encoding acute-phase proteins [19,20]. STAT3 plays a critical role in responses to many cytokines and growth factors, including γc cytokines (IL-2, IL-7, IL-9, IL-15, and IL-21), IL-6/gp130 cytokines (IL-6, IL-11, IL-27, IL-31, CNTF, CT-1, oncostatin M, and leukemia inhibitory factor), type 1 and type 2 IFNs (IFNα, IFNβ, and IFNγ), IL-10 family cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, and IL-29), receptor tyrosine kinases (EGF, FGF, Flt3 ligand, GH, IGF1, M-CSF, and PDGF), as well as IL-5, IL-12, IL-23, G-CSF, leptin, and PAF. A study of a null mutation in the STAT3 gene in mice demonstrated that STAT3 was essential for the survival of the embryo around the time of implantation [31]. Studies of mice with tissue-specific deletions of *STAT3* have shown STAT3 to play a crucial role in cell proliferation, survival, migration, apoptosis and inflammation in various tissues, organs and cells, including skin, respiratory epithelium, thymic epithelium, liver, mammary glands, neurons, lymphocytes, and macrophages [32]. Many of the clinical signs of type 1 HIES patients therefore probably reflect defects of the diverse functions of STAT3 *in vivo*.

Pathogenesis

Our knowledge of the pathogenesis of HIES in patients with *STAT3* mutations remains limited, despite the discovery of the molecular etiology of HIES. Several major questions remain unanswered: first, What is the molecular mechanism underlying atopic dermatitis and high serum IgE levels in these patients? second, What is the molecular mechanism underlying the observed skeletal abnormalities?

Staphylococcal infection of the skin and lung

T_H17 cells are a newly identified subset of helper T-cells associated with the exacerbation of various autoimmune disorders, including inflammatory bowel disease, multiple sclerosis, psoriasis, and rheumatoid arthritis [33–41]. T_H17 cells produce T_H17 cytokines, including IL-17 (IL-17A), IL-17F, and IL-22. Their functions in humans remain unclear, but there is evidence to suggest that T_H17 cells play a crucial role in the recruitment of neutrophils and production of the antimicrobial peptides β-defensin 2 (BD2) and BD3 by epithelial and endothelial cells.

Recent studies have demonstrated defects in the differentiation of T_H17 cells in patients with HIES [27•,29,42•,43•]. The occurrence of staphylococcal infections in HIES patients suggests that T_H17 cells play a crucial role in protecting against *S. aureus*, not only in mice but also in humans. However, these infections are usually confined to the skin and lungs. We therefore tested the hypothesis that the cells in the skin and lung have unique features. We found that primary human keratinocytes and bronchial epithelial cells were more dependent on T_H17 cytokines than other types of cell for the production of antistaphylococcal factors, including the neutrophil-recruiting chemokines and anti-bacterial peptides [44•]. T-cells from HIES patients, despite having defects in the production of T_H17 cytokines, produced other proinflammatory cytokines, including IL-1β and TNFα, in normal amounts. These cytokines were not sufficient to trigger antistaphylococcal factor production in keratinocytes and bronchial epithelial cells, but the production of these factors was induced in fibroblasts and endothelial cells. These findings provide a possible molecular explanation for the apparent paradox of systemic T_H17 deficiency in HIES patients with staphylococcal infections restricted to the skin and lungs. They also provide interesting insight into tissue-specific immunity. Epithelial cells are constantly exposed to environmental agents. They have evolved to respond only weakly to the

first signal — the classical proinflammatory cytokines produced by innate immune cells — but respond more strongly to a combination of this first signal and a second signal provided by T-cells.

Antigen-specific antibody production

HIES patients have normal serum IgM, IgG, and IgA levels, but most have defects of various types in the antigen-specific antibody response to immunization [1]. They also suffer from infections with encapsulated bacteria [45–48]. IgG subclass deficiencies have been reported in some patients [1]. Most patients with HIES with *STAT3* mutations have smaller than normal numbers of circulating memory B cells [49].

Mice lacking *STAT3* expression in B cells display normal B-cell development, normal baseline serum antibody levels of IgM, IgG, and IgA and produce normal T-independent (TI) and T-dependent (TD) IgM, IgA, and IgE responses, but have defective TD IgG responses [50]. Germinal center formation, isotype switching, and the generation of memory B cells all occur normally in STAT3-deficient murine B cells. There are several possible reasons for the difference in phenotype between humans and mice: first human mutations are *dominant negative*, but not null; second, mutations in humans are expressed not only in B cells, but also in other cell types, with possible indirect effects on B-cell differentiation. Alternatively, there may be a species-specific difference in the requirement for STAT3 between humans and mice.

Skeletal manifestations

STAT3 plays important roles in the differentiation of both osteoblasts and osteoclasts *in vitro*, and mice lacking STAT3 in osteoblasts or osteoclasts have an osteoporotic phenotype [51,52]. In humans, osteoclasts generated from peripheral blood monocytes from HIES patients with *STAT3* mutations and display higher levels of bone-resorption activity than those from control subjects [12••]. These observations are consistent with the osteoporosis observed in HIES patients, but it remains unclear why these patients also display the retention of deciduous teeth, which would be expected to result from the impairment of osteoclast activity.

Hyper-IgE-emia

Serum concentrations of IgE are extremely high in patients with HIES, usually at least a hundred times higher than those in normal individuals (>2000 IU/ml). The molecular mechanism of hyper-IgE-emia remains unknown. Murine IL-21 inhibits IgE production by B cells, but human IL-21 acts in synergy with IL-4 to upregulate IgE production [53]. Unlike IgG-producing plasma cells, IgE-producing plasma cells are excluded from the germinal center [54]. In the absence of Bcl-6, a transcriptional repressor required for germinal center formation, class-switching to IgE in response to IL-4 is upregulated because of a loss of repression of IL-4/STAT6 [55]. IL-21 may be involved in the trafficking of developing B cells in the germinal center [56–59]. IL-6/STAT3 signaling promotes the differentiation of naive T-cells into helper T-cells capable of assisting B cells, in a process independent of follicular helper T-cell differentiation [60]. It is not yet clear how these pieces of the puzzle fit together to explain the extremely high serum IgE levels in HIES patients. This is clearly one of the most important unresolved issues and its resolution might open up new possibilities for regulating serum IgE levels in common atopic disorders.

Conclusion

In the past three years, we have witnessed major progress toward the identification of the molecular causes of HIES. The identification of a patient with TYK2 deficiency revealed the critical functions of TYK2 in the transduction of multiple cytokine signals involved in innate and acquired immunity, and demonstrated the greater importance of TYK2 functions in humans than in mice. The identification of TYK2 deficiency as an etiology of type 2 HIES led to the identification of *dominant-negative STAT3* mutations as a major molecular cause of type 1 HIES.

This discovery has improved our understanding of the crucial roles played by STAT3 in protection against extracellular pathogens and Th17 cell differentiation. Studies of patients with HIES provide further evidence of the power of 'experiments of nature' for dissecting the complex human immune system. Answers to most of the unresolved questions will probably be found in the near future, providing further insight into the various aspects of the human immune system involved in HIES development. This progress should eventually lead to improvements in the treatment for patients.

Conflict of interest

The authors have no conflicting financial interests.

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Defects in Jak–STAT-mediated cytokine signals cause hyper-IgE syndrome: lessons from a primary immunodeficiency

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REVIEW

Abstract

Hyper-IgE syndrome (HIES) is a primary immunodeficiency characterized by atopic manifestations and susceptibility to infections with extracellular bacteria and fungi, which frequently occur in the skin and lung. Atopic manifestations in HIES include extremely high serum IgE levels, eczema and eosinophilia. Most of the extracellular bacterial infections are associated with disproportionally milder inflammation than normal, which was originally described as having a 'cold abscess'. Non-immunological abnormalities are also observed in most patients with HIES, including a distinctive facial appearance, scoliosis, hyper-extensive joints and retained primary teeth. Recent studies have demonstrated that hypomorphic mutations in signal transducer and activator of transcription 3 result in the classical multisystem form of HIES, whereas a null mutation in tyrosine kinase 2 causes the autosomal recessive form of HIES that is associated with viral and mycobacterial infections. Analyses of cytokine responses in both types of HIES have revealed defects in signal transduction for multiple cytokines including IL-6 and IL-23, leading to impaired T_h17 function. These results suggest that the defect in multiple cytokine signals is the molecular basis of the immunological and non-immunological abnormalities in HIES and that the susceptibility to infections with extracellular bacteria and fungi in HIES might be associated with the defect in T_h17 cell differentiation.

Introduction

Primary immunodeficiencies are genetically determined immune disorders. Research into these disorders, in conjunction with the study of 'knockout' mice, has led to remarkable progress in our understanding of the *in vivo* function of certain genes expressed in the immune system. Identification of the molecular origin of primary immunodeficiencies also provides benefit for the patients, resulting in earlier diagnosis and better treatment. The number of genes identified as responsible for primary immunodeficiencies has recently grown tremendously, reflecting the explosive expansion of knowledge in this field (Table 1). In this article, we will first briefly review the contribution of one primary immunodeficiency (agammaglobulinemia) to our understanding of the human immune system and later focus on another primary immunodeficiency, hyper-IgE syndrome (HIES).

Agammaglobulinemia

The first primary immunodeficiency disorder was reported in 1952, when Ogden Bruton described an 8-year-old boy who experienced recurrent, life-threatening pneumococcal pneumonia and septicemia (1). The patient was able to survive, thanks to the discovery and clinical use of antibiotics at that time, which enabled the identification of the disorder. Using a newly developed serum electrophoresis technique, Bruton identified that the boy was deficient in gammaglobulin. With the treatment using intramuscularly injected gammaglobulin isolated from healthy individuals, the patient's susceptibility to bacterial infections was dramatically decreased. The disorder described in the report is now called X-linked agammaglobulinemia (XLA). Patients with XLA can remain healthy during the first few months of life, thanks to maternally transmitted IgG antibodies. Thereafter, the patients repeatedly suffer from infections with extracellular bacteria such as pneumococci and haemophilus unless prophylactic antibiotics or gammaglobulin therapy is initiated. Concentrations of Igs of all the isotypes are very low in the patients, and circulating B cells are almost absent. In the bone marrow, the number of pro-B cells is normal, but that of pre-B cell is

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Table 1. Classification of primary immunodeficiencies

A. Combined T and B cell immunodeficiency
1. T-B– SCID (RAG1/2, Artemis, ADA)
2. T-B+ SCID (γc, Jak3, IL-7Rα, CD45, CD3δ/ε/ξ)
3. Omenn syndrome (hypomorphic mutation in Rag1/2, Artemis, IL-7Rα)
B. Predominant antibody deficiencies
1. Agammaglobulinemia (Btk, μ heavy chain, λ5, Igα, Igβ, BLNK)
2. Common variable immunodeficiency (ICOS, CD19)
3. Hyper-IgM syndrome (CD40L, CD40, AID, UNG)
C. Other well-defined immunodeficiency syndromes
1. HIES (STAT3, TYK2)
2. Wiskott–Aldrich syndrome (WASP)
3. DNA repair defects (ATM, MRE11, NBS1, BLM)
D. Diseases of immune dysregulation
1. Familial hemophagocytic lymphohistiocytosis (PRF1, MUNC13D, STX11)
2. X-linked lymphoproliferative syndrome (SH2D1A, XIAP)
3. Syndrome with autoimmunity (Fas, FasL, CASP10, CASP8, AIRE, FOXP3)
E. Congenital defects of phagocytes
1. Severe congenital neutoropenia (ELA2, GFI1, G-CSFR)
2. Kostman disease (HAX1)
3. Leukocyte adhesion deficiency (ITGB2, FUCT1)
F. Defects in innate immunity
1. Ectodermal dysplasia with immunodeficiency (NEMO)
2. IRAK4 deficiency (IRAK4)
3. Herpes virus encephalitis (UNC93B1, TLR3)
G. Autoinflammatory disorders
1. Familial Mediterranean fever (MEFV)
2. Hyper-IgD syndrome (MVK)
3. Muckle–Wells syndrome (CIAS1/NALP3)
H. Complement deficiencies (C1q, C1r, C1s, C4, C2, C3, C5, C6, C7, C8a, C8b, C9, factor I/H/D)

Some examples of primary immunodeficiency and its molecular origins from the World Health Organization classification (29), the responsible molecules for each category are shown in parenthesis. ADA, adenosine deaminase; AID, activation-induced cytidine deaminase; AIRE, autoimmune regulator; ATM, ataxia telangiectasia mutated; BLM, Bloom's syndrome protein; BLNK, B cell linker protein; Btk, Bruton tyrosine kinase; CASP, caspase; CIAS1, cold-induced autoinflammatory syndrome; ELA, neutrophil elastase; FOXP3, forkhead box P3; FUCT1, GDP-fucose transporter 1; GFI1, growth factor independent 1; HAX1, HSLS1-associated protein X1; ICOS, inducible costimulatory; ITGB2, integrin β-2; IRAK4, IL-1 receptor-associated kinase; MEFV, familial Mediterranean fever; MVK, mevalonate kinase; Mre11, meiotic recombination 11; NALP3, Nacht domain-, leucine-rich repeat and PYD-containing protein 3; NBS1, Nijmegen breakage syndrome 1; NEMO, NF-kB essential modulator; PRF1, perforin 1; RAG, recombinase-activating gene; SH2D1A, SH2 domain protein 1A; STX11, syntaxin 11; TLR, Toll-like receptor; UNC93B1, Unc-93 homolog B1; UNG, uracil-DNA glycosylase; WASP, Wiskott–Aldrich protein; XIAP, X-linked inhibitor of apoptosis protein.

severely decreased. Tonsils are usually very small, and lymph nodes are non-palpable because of the absence of germinal centers in the lymphoid tissues.

Patients with agammaglobulinemia are susceptible to encapsulated bacteria, including *Streptococcus pneumonia* and *Haemophilus influenza*. In contrast, they are not susceptible to infections by fungi, coliforms, intracellular bacterial and many kinds of viruses with the exceptions of hepatitis viruses and enteroviruses. This differential susceptibility of agammaglobulinemic patients to the microbial pathogens and the beneficial effects of therapeutic gammaglobulin highlighted a crucial role of antibodies in protection against encapsulated bacteria. Furthermore, before the discovery of the primary immunodeficiencies, it was assumed that infections are attributable to excessive exposure to infectious agents or unusual properties of the infectious organisms involved. Thus, the recognition of the first human primary immunodeficiency disease has set the stage for an exponential increase in information about the functions of the various components of the human immune system and its defects as a result of 'experiments of nature' (2).

In 1993, two groups independently discovered a gene responsible for XLA, now called Bruton agammaglobulinemia tyrosine kinase. One group positionally cloned (see below) the responsible gene in human (3). The other group identified it as a new B cell-specific tyrosine kinase that is important in murine B cell signaling (4). The group next realized that the gene was located on the X chromosome. When the human gene counterpart was cloned, alterations in the nucleotide sequence in the tyrosine kinase were identified in patients with XLA.

Agammaglobulinemia is one of the prototypes of primary immunodeficiencies, and the process of identification of the molecular origin provides a typical example for many other primary immunodeficiencies (5–8). In most cases, a molecular origin is identified utilizing both genetic and immunological clues. Theoretically, there are two approaches for the identification of the genetic determinant for primary immunodeficiencies. One is a classical position-dependent approach (positional cloning) and the other is a position-independent approach. In positional cloning, the genes in the candidate region are selected for further evaluation on the basis of the expression pattern and/or function of the molecules in the region; here, phenotypic homology between humans and mice provides valuable clues toward identifying genes. Positional information reduces the number of possible candidate genes enormously. At present, this is very important because our ability to predict appropriate candidate genes is very limited.

History of HIES

HIES (also called Job's syndrome; #147060 and #243700 in the Online Mendelian Inheritance in Man human genetic disease database) is another primary immunodeficiency syndrome, one of the members of the 'other well-defined immunodeficiency syndrome' category in the classification defined by the World Health Organization (Table 1). This disease was first described in 1966 by Davis and Wedgwood (9). Two patients reported by them had recurrent staphylococcal skin infections that lacked the features of typical inflammation including redness and warmness, and therefore Davis and Wedgwood coined the phrase 'cold abscess' for the phenomenon. The syndrome was further characterized by Buckley *et al.* (10), who noted that the recurrent staphylococcal skin infections and cold abscess formation in HIES were associated with severe dermatitis and highly elevated serum IgE levels. Later study further characterized the multisystem nature of HIES, in which the manifestations are not only restricted to the immune system but also extend to skeletal and dental abnormalities such as a unique facial appearance, scoliosis, osteoporosis, hyper-extensive joints

and retained primary teeth (11). In 2004, a novel form of HIES was identified in consanguineous families, suggesting the presence of autosomal recessive (AR) HIES (12).

Clinical characteristics of HIES

As well as by high levels of serum IgE and recurrent bacterial infections, HIES is characterized by eczema similar to that found with atopic dermatitis (13, 14). The levels of IgE in the serum are extremely high—usually at least 100 times higher than those of normal individuals. The atopic dermatitis-like eczema usually starts in the neonatal period—much earlier than the onset of atopic dermatitis, which is a common skin disorder. In HIES, infections with extracellular bacteria generally commence in infancy and mainly involve the skin and lung. The most common bacterium involved in the infections is *Staphylococcus aureus*, although *S. pneumoniae*, *H. influenza* and enteric gram-negative bacteria are occasionally isolated in some infectious episodes of HIES. Fungal infections including mucocutaneous candidiasis and lung aspergillosis are also common in HIES.

Most cases of HIES are sporadic, but some familial cases of HIES have been reported, either with an autosomal dominant (AD) (11) or with an AR (15) mode of inheritance. Skeletal and dental abnormalities are observed in sporadic cases and familial cases with the AD form, but not those with the AR form that is characterized by severe, recurrent viral infections, extreme eosinophilia and neurological complications. From both clinical and etiological points of view, we classify HIES into two groups, type 1 and type 2, irrespective of modes of inheritance. Type 1 HIES displays abnormalities in multiple systems of the body including the skeletal and dental systems, whereas type 2 HIES shows abnormalities confined to the immune system (Table 2).

Type 1 HIES

This group of HIES represents the most common form of HIES, including both sporadic and familial AD inheritance (11). In addition to the recurrent staphylococcal skin and pulmonary infections, atopic dermatitis and elevated serum IgE found in almost all the patients with HIES, patients with type 1 HIES display skeletal, dental and connective tissue manifestations. By the age of 16 years, all the patients show a distinctive facial appearance: coarse texture of facial skin, asymmetric facial appearance, prominent forehead, deep-set eyes, broad nasal bridge and bulky nasal tip. In this type of HIES, pneumonia is frequently followed by the formation of pulmonary cysts, most likely due to the impaired remodeling capability of the lung. Pulmonary cysts are frequently complicated by the superinfection of *Aspergillus* and multi-drug-resistant *Pseudomonas aeruginosa*, which is a serious problem in the morbidity and the mortality of patients with HIES (16).

Table 2. A classification of HIES

HIES type	Inheritance	Distinguishing clinical findings
Type 1 (multisystem)	Sporadic (>90% of cases)	Skeletal and dental abnormalities (characteristic face, fracture with minor trauma, retained primary teeth, scoliosis and hyper-extensibility)
	Familial with AD inheritance (rare)	Pulmonary cyst (pneumatocele)
Type 2	Familial with AR inheritance	Severe viral infections (herpes simplex virus, molluscum contagiosum) Central nervous system involvement (?) Mycobacterial infections (some) No pulmonary cyst No skeletal manifestations

Type 2 HIES

This group of HIES was reported relatively recently; six consanguineous families from Turkey and Mexico were suggested to carry the AR form of HIES (12). More than two patients were present in five out of the six pedigrees. The patients in these families did not show any apparent abnormalities in their skeletal and dental systems but suffered from recurrent and severe infections with *S. aureus*, *S. pneumoniae* or *H. influenzae*, as observed in type 1 HIES. Notably, most of the patients with type 2 HIES also suffered from recurrent viral infections such as chronic refractory molluscum contagiosum and herpes simplex virus infections, which were not identified in type 1 HIES. Furthermore, no pulmonary cyst was found in any of the patients with type 2 HIES, unlike in the patients with type 1 HIES. Seven out of 13 patients with type 2 HIES had neurological complications, and four of them died of the neurological symptoms. The origin of the neurological complications might be due to either primary to the type 2 HIES or secondary to latent infections in the central nervous system.

Diagnosis of HIES

Clinical diagnosis of HIES is based on the criteria established by Grimbacher *et al.*, which scores clinical findings such as the number of skin abscesses (more than four times in a lifetime is 8 points) and pneumonia (more than three episodes in a lifetime is 8 points), parenchymal lung abnormalities (pneumatocele is 8 points), characteristic facial appearance (if typical, 5 points), newborn rash (if present, 4 points), hyper-extensibility (if present, 4 points) as well as laboratory findings such as high serum IgE concentrations (>2000 IU ml^{-1}, 10 points) and eosinophilia (>800 mm^{-3}, 6 points) (17). We clinically diagnose HIES if the score of the patient is above 40 points. Although not included in this scoring system, one of the unique characteristics of the HIES is the apparent lack of classical inflammatory responses, which might be included in the scoring system for the future.

Defining the etiology of HIES

Early findings

The origin and the molecular pathology of HIES remained an enigma in spite of >40 years of extensive research, until the recent discovery of causative genes. Previous study by

Hill *et al.* (18) suggested that impaired neutrophil chemotaxis caused the susceptibility to extracellular bacterial infection; however, it was later realized that the defect was not consistently present in patients with HIES. After the identification of T_h1 and T_h2 cells in mice, many researchers investigated T_h1 and T_h2 cell differentiation in patients with HIES. Although some studies suggested a decreased T_h1 cytokine production and a skewing toward T_h2 cytokine production (19–21), the results were inconsistent from study to study and did not provide sufficient clues to identify the molecular origin of HIES. It should be noted that the patients with defects in IL-12 signaling manifest none of the common features of HIES, suggesting that the simple reduction of IFN-γ in response to IL-12 is unlikely to cause HIES (22).

More than 250 HIES patients have been reported in the literature. An HIES patient with mental retardation was identified to have a deletion of 15–20 cM on chromosome 4q. Linkage analysis of 19 families with 57 HIES patients demonstrated linkage to the proximal arm of chromosome 4q; however, 6 of the 19 families did not demonstrate linkage to this region of chromosome 4q. Another study suggested a polymorphism in the IL-4R (Q576R) linked to HIES; however, later study showed no correlation of the Q576R alleles with the HIES phenotype. Collectively, although many attempts were made to identify the molecular origin of HIES, they were not successful until we identified an AR-HIES patient associated with viral and intracellular bacterial infection 3 years ago.

Tyrosine kinase 2 deficiency as the genetic origin for a subpopulation of type 2 HIES

The first genetic origin identified in HIES was a null mutation of tyrosine kinase 2 (*TYK2*) (23). The identification of *TYK2* as the causative gene was suggested following the observations that a patient with HIES was susceptible to intracellular bacterial infections and had defective signaling by IL-12 and IFNα. Tyk2 is one of the founding members of Janus kinase family (Jaks) (24, 25), which transduces signals initiated by most of cytokines (26–28). Jaks constitutively associate with the cytoplasmic domains of cytokine receptors, which lack intrinsic kinase activity, and upon ligand binding, phosphorylate receptor subunits. This recruits signal transducers and activators of transcription (STATs) and other adapters and signaling molecules. Jaks also phosphorylate and activate STATs, promoting nuclear translocation and transcription of STAT-responsive genes.

The patient had a homozygous 4-bp deletion in the coding region of *TYK2* gene, resulting in a premature stop codon and the absence of TYK2 protein. The parents of the patient were consanguineous, and both were heterozygous for the mutation, establishing the AR inheritance of the TYK2 deficiency in the patient. The TYK2-deficient patient displayed typical phenotypes of type 2 HIES, including elevated serum IgE, atopic dermatitis, infections with extracellular bacteria and viral infections but no skeletal and dental abnormalities. Now this disorder is termed AR-HIES associated with susceptibility to virus and intracellular bacterial infections (29).

Blood cells from the patient showed severe defects in response to multiple cytokines including type I IFN (IFNα and IFNβ), IL-6, IL-10, IL-12 and IL-23 (Fig. 1). Importantly, the cytokine signals were successfully restored by transducing the intact TYK2 gene *in vitro*. Thus, the TYK2 deficiency is likely to account for the clinical manifestations of the patient (Fig. 2). The susceptibility of the patient to viral infections could be explained by the defects in type I IFN signaling. IFNα-induced tyrosine phosphorylation of Jak1, STAT1 and STAT2 was completely abrogated in the human TYK2-deficient cells. Furthermore, no inhibition in the replication of herpes simplex virus was observed in the patient's cells when pre-treated with IFNα. One of the features characteristic of human TYK2 deficiency is susceptibility to intracellular bacterial infection such as mycobacteria and salmonella. The absence of TYK2 resulted in defects of both IL-12 and type I IFN signaling, which lead to impaired T_h1 differentiation and IFN-γ production. This defect likely accounts for the patient's susceptibility to intracellular bacterial infections as reported in other disorders with the defects in the IL-12 and IFN-γ signaling circuit (30). When the patient's naive CD4

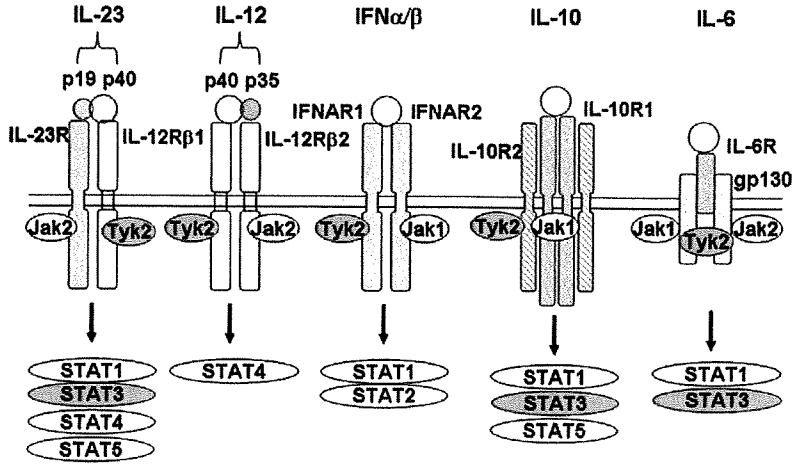

Fig. 1. Surface cytokine receptors and their associated intracellular Jaks (Jak1, Jak2 and Tyk2) and STATs.

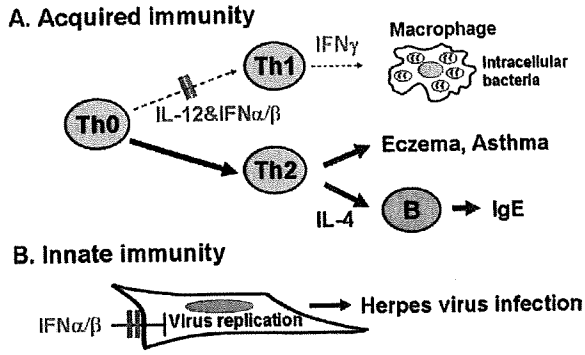

Fig. 2. Pathogenesis of TYK2 deficiency. (A) In acquired immunity, the absence of TYK2 results in defects of both IL-12 and type I IFN signaling, which lead to impaired T_h1 differentiation and IFN-γ production. This defect likely accounts for the patient's susceptibility to intracellular bacterial infections. The patient's naive CD4 T cells showed enhanced T_h2 differentiation and over-production of IL-4 in addition to impaired T_h1 differentiation, reflecting the T_h2-dominant phenotypes of the patient, such as eczema, asthma and elevated serum IgE level. (B) In innate immunity, the absence of TYK2 results in defective type I IFN signaling, resulting in susceptibility to herpes virus infections.

T cells were cultured *in vitro*, enhanced T_h2 differentiation was observed in addition to the impaired T_h1 differentiation, reflecting the T_h2-dominant phenotypes of the patient, such as atopic dermatitis, asthma and elevated serum IgE levels. Since IL-10 plays a critical role in peripheral T cell tolerance to allergens, it might be reasonable to speculate that allergic symptoms in the TYK2 deficiency were associated, at least in part, with the defective IL-10 signaling.

The functional importance of the Jaks was demonstrated by studies in cell lines defective in cytokine signaling and confirmed by the establishment of knockout mice (31–36). Although TYK2 was originally described as essential for the type I IFN signaling in a human fibroblast cell line (24, 25), the establishment of Tyk2-deficient mice revealed that Tyk2 was not absolutely essential for responses to type I IFN in mice (37, 38). Tyk2−/− mice displayed a lack of responsiveness to a small amount of IFNα, but a high concentration of IFNα could fully transduce its signals even in the absence of Tyk2. Thus, the function of Tyk2 in mice appears to be compensated for *in vivo*, partly by other kinases, most likely other Jaks, which is not the case in humans.

Hypomorphic mutations in STAT3 cause type 1 HIES

The identification of the TYK2 deficiency in a patient with type 2 HIES suggested to us that a similar defect in cytokine signaling might be observed in the type 1 HIES. To explore this possibility, we first examined the responses to IL-6, IL-10, IL-12 and IFNα of peripheral blood cells from typical type 1 HIES patients. IgM secretion from the patients' B cells after stimulation with EBV in the absence or the presence of IL-6 demonstrated that the signaling of IL-6 was impaired in type 1 HIES. In addition, the suppression of LPS-induced production of tumor necrosis factor (TNF)α by IL-10 was also impaired in the patients. Thus, both IL-6 and IL-10 signaling pathways were defective in type 1 HIES, as in TYK2 deficiency. In contrast, neither IL-12 nor IFNα signaling was impaired in patients with type 1 HIES.

In a survey of candidate molecules involved in both IL-6 and IL-10 signaling, but not involved in the type I IFN and IL-12 signaling, we identified heterozygous, dominant negative, mutations in the DNA-binding domain of STAT3 in the patients (39). The DNA-binding domain of STAT3 is highly conserved among different species in its amino acid sequence, and the alterations were not found in 1000 unrelated healthy individuals analyzed. STAT3 is located on human chromosome 17q21 but not 4q that was reported to contain a disease locus for familial AD-HIES (17). None of the eight HIES patients in our 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. Therefore, the mutations are likely to have occurred *de novo* in the patients with this form of HIES.

Later study further identified mutations in the STAT3 gene in most of the patients with type 1 HIES, including the patients described in the first report of HIES (16, 40). The position of mutations was located in the DNA-binding domain of STAT3 in the first eight patients, which extended to the Src homology 2 domain and the transactivation domain (41, 42). The clinical characteristics of the patients have been indistinguishable between those with mutations in the DNA-binding domain and those with mutations in other domains of STAT3 molecule, and there seems no clear phenotype–genotype relationship in the HIES.

STAT3 is a transcription factor, which binds to the STAT3-responsive elements in the promoters of various genes including acute-phase proteins (43–46). STAT3 plays a critical role in responses to many cytokines, including IL-6, IL-10, IL-22, IL-23 and IL-27 (47–49). A null mutation of the STAT3 gene in mice demonstrated that STAT3 was essential for embryonic survival near the time of implantation (E6.5–E7.5) (50). It is reasonable to speculate that the difference between AD inheritance of hypomorphic mutations in human and early embryonic lethality of a null mutation in mice is due to the fact that the human mutations have residual STAT3 activity. It is, however, still possible that human and mouse STAT3 have distinct functions. Future study should clarify this issue. Mice with tissue-specific deletion of STAT3 were established, which demonstrated the critical role of STAT3 in cell migration, survival, proliferation, apoptosis and inflammation in each tissue, including skin, mammary glands, liver, thymic epithelium, respiratory epithelium, neurons, lymphocytes and macrophages (51). Thus, it is likely that wide range of manifestations in type 1 HIES patients corresponds to the diverse function of STAT3 *in vivo* in human.

Molecular pathogenesis and T_h17 defect in HIES

Although the molecular origin of the HIES has been identified, we still know very little about the molecular pathogenesis of the HIES. There are at least four important questions: what is the molecular mechanism of skeletal and dental manifestations; why do patients with HIES display little or no inflammatory response in spite of severe infections; why do patients with HIES have atopic manifestations and why do

patients with HIES suffer from extracellular bacterial infections confined to the skin and lung?

STAT3 plays important roles in the differentiation of both osteoblasts and osteoclasts *in vitro*, and mice deficient for STAT3 in osteoblasts show an osteoporotic phenotype (52). When osteoclasts were generated from peripheral blood monocytes in culture with M-colony-stimulating factor 1 and receptor activator of nuclear factor κB ligand (also known as TNF superfamily, member 11), those from HIES patients with STAT3 mutations showed higher bone resorption activity compared with those from control subjects (39). This may reflect the skeletal and dental abnormalities observed in patients with HIES.

Another remarkable clinical feature of HIES is that patients are often afebrile and doing well, despite serious pneumonia or dermal pathology (referred as cold abscess) (9). Moreover, the acute-phase responses, such as increased serum C-reactive protein levels during infections, were diminished in the patients. STAT3 was originally identified as a protein binding to the IL-6-responsive element in the genes encoding hepatic acute-phase proteins (43), and the liver-specific inactivation of STAT3 leads to an impaired acute-phase response in mice. Thus, the apparent lack of classical inflammatory responses in HIES patients could be attributed to defective signaling of IL-6.

We have no clear explanation for the mechanism underlying the high levels of serum IgE in patients with HIES. We and others considered the possibility that signaling a defect of IL-21 might be the origin of high levels of IgE in the serum. Surprisingly, a recent report indicated that although IL-21 in mice suppresses IL-4-induced IgE production, IL-21 in human induces IgE production by CD40 ligand-stimulated naive B cells (53). Future study should clarify these unanswered questions.

STAT3 plays a critical role in T_h17 development (54), and IL-17 produced by T_h17 cells is protective in host defense against extracellular bacteria (55–57). IL-22 stimulates cells in the skin and respiratory systems to produce β-defensins through STAT3 activation (58, 59). Thus, the susceptibility to extracellular bacterial infection could be attributed, at least in part, to defects in T_h17 development and IL-22 signaling. Indeed, defective T_h17 development and function in patients with HIES due to mutations in STAT3 was recently demonstrated (60–62). Future studies should address why and how defective T_h17 cells specifically cause the susceptibility to staphylococcal infections in the skin and lung.

Conclusion and perspective

The identification of human TYK2 deficiency revealed the critical functions of TYK2 in the transduction of multiple cytokine signals involved in the innate and acquired immunity and demonstrated that TYK2 has broader and more profound functions in humans compared with mice. The establishment of TYK2 deficiency as an etiology for a rare form of AR-HIES associated with susceptibility to viral and intracellular bacterial infections suggested us that the classic type 1 HIES is also associated with the deficiency in multiple cytokine signals; which led to the identification of dominant-negative STAT3 as a major molecular origin of type 1 HIES. These studies highlight the multiple and critical roles played by STAT3 in humans *in vivo* and provide important information for the physician taking care of patients with HIES.

The identification of STAT3 as a major causative gene for type 1 HIES enables us to definitively diagnose the HIES patients very early in life. The key feature for early diagnosis used to be newborn rash, which is frequently associated with the susceptibility to staphylococcal infection and high serum IgE levels. Earlier definitive diagnosis at the DNA level facilitates the early start of prophylactic antibiotics, which is likely to prevent the pneumatocele formation. Preventive action should improve the quality of life of the HIES patients. Although our current attempts to improve treatment for HIES mainly focuses on early diagnosis and the prevention of disease progression, future treatment options should include stem cell transplantations and gene-targeted therapies.

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Abbreviations

AD	autosomal dominant
AR	autosomal recessive
HIES	hyper-IgE syndrome
Jaks	Janus kinase family
STAT	signal transducer and activator of transcription
TNF	tumor necrosis factor
TYK2	tyrosine kinase 2
XLA	X-linked agammaglobulinemia

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Notch Activation Induces the Generation of Functional NK Cells from Human Cord Blood CD34-Positive Cells Devoid of IL-15[1]

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The development of NK cells from hematopoietic stem cells is thought to be dependent on IL-15. In this study, we demonstrate that stimulation of human cord blood CD34$^+$ cells by a Notch ligand, Delta4, along with IL-7, stem cell factor, and Fms-like tyrosine kinase 3 ligand, but no IL-15, in a stroma-free culture induced the generation of cells with characteristics of functional NK cells, including CD56 and CD161 Ag expression, IFN-γ secretion, and cytotoxic activity against K562 and Jurkat cells. Addition of γ-secretase inhibitor and anti-human Notch1 Ab to the culture medium almost completely blocked NK cell emergence. Addition of anti-human IL-15-neutralizing Ab did not affect NK cell development in these culture conditions. The presence of IL-15, however, augmented cytotoxicity and was required for a more mature NK cell phenotype. CD56$^+$ cells generated by culture with IL-15, but without Notch stimulation, were negative for CD7 and cytoplasmic CD3, whereas CD56$^+$ cells generated by culture with both Delta4 and IL-15 were CD7$^+$ and cytoplasmic CD3$^+$ from the beginning and therefore more similar to in vivo human NK cell progenitors. Together, these results suggest that Notch signaling is important for the physiologic development of NK cells at differentiation stages beyond those previously postulated. *The Journal of Immunology,* **2009, 182: 6168–6178.**

Natural killer cells are critical for host immunity because they rapidly mediate cellular cytotoxicity against pathogen-infected or malignantly transformed cells and produce a wide variety of cytokines and chemokines that influence other components of the immune system. Unlike other lymphocytic lineages, however, the continuous staging scheme of human NK cell development in vivo has yet to be elucidated (1). One reason for this may be the difficulty in closely correlating our knowledge of mouse NK cell biology with human NK cell biology (2), because mouse NK cells do not express a homolog of CD56, which is the marker most representative of human NK cells; instead, the most widely used markers of NK cells in various mouse strains are NK1.1 and DX5, mouse-specific Ags. Among the molecules involved in NK cell development, IL-15 has a particularly important role. For example, IL-15-deficient mice lack NK1.1$^+$ cells (3), indicating that IL-15 is essential for NK cell development in mice. The requirement of IL-15 for mouse NK cell development has also been demonstrated by other studies (4, 5). In humans, IL-15 is considered to be required for in vitro NK cell development and virtually most current protocols for human NK cell differentiation culture depend on IL-15. IL-15-independent NK cell differentiation has been reported in which human cord blood (CB)[9] cells are cocultured with murine stromal cell lines (6). Signaling, however, substituting IL-15 signaling that is responsible for the NK cell differentiation in this culture system was not described.

NK cells are thought to be derived from hematopoietic stem cells through a T/NK precursor stage. The Notch signaling pathway influences cell fate decisions in numerous cellular systems,

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[9] Abbreviations used in this paper: CB, cord blood; cy, cytoplasmic; FL, Fms-like kinase 3 ligand; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester; CMA, concanamycin A.

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including various hematopoietic and immune cells (7–9). To date, four Notch receptors (Notch1– Notch4) and at least four Notch ligands (Delta1, Delta4, Jagged1, and Jagged2) have been identified in mammals. Signaling through Notch1 is crucial in the early stages of T cell development (10–12). In culture, ligand-induced Notch signaling drives human CB CD34$^+$ cells to differentiate into T/NK cell precursors (13). Furthermore, Notch signaling drives the T/NK precursors toward differentiation into T and NK cells, although the results for the NK cells are controversial. For example, inhibition of Notch signaling suppresses T cell development and stimulates NK cell development (14–16), whereas activation of Notch signaling contributes to the efficient development of NK cells in mice (17, 18) and humans (19). It is not concluded, however, whether Notch signaling is involved in the function of NK cells or whether IL-15 is necessary for NK cell development in culture.

In this report, to gain further insight into the physiologic significance of Notch signaling in NK cell development, we examined whether IL-15 is dispensable for the generation of functional NK cells and whether Notch signaling has a role in the later stages of NK cell development. Our results indicated that Notch signaling, but not IL-15 stimulation, was essential for inducing CD34$^+$ cells to give rise to CD7$^+$ and cytoplasmic (cy) CD3$^+$ cells that express CD56 in stroma-free culture. Surprisingly, cells cultured with Delta4-coated plates, but lacking IL-15 in the medium, were functional NK cells with cytotoxic activity. IL-15, along with Delta4, further augmented NK cell activity and phenotypic maturation. The addition of IL-15 without exogenous Notch ligand, however, did not allow CD34$^+$ cells to take a NK cell developmental pathway resembling physiologic NK cell precursors. Notch signaling might have a significant role in the development of NK cells in vivo.

Materials and Methods

Reagents and Abs

Recombinant human Delta4-Fc chimeric protein was generated as described previously (20). Recombinant human IL-7 and IL-15 were purchased from R&D Systems. Human stem cell factor and human Fms-like kinase 3 ligand (FL) were a gift from Amgen. Human IL-6/IL-6 receptor fusion protein (FP6) and human thrombopoietin were provided by Kirin Pharma. Anti-IL-15 Ab (MAB2471) and isotype control mouse IgG1 were purchased from R&D Systems. Anti-CD3 (UCHT1), CD8 (SK1), CD14 (M5E2), CD44 (G44-26), CD45 (HI30), CD45RA (HI100), CD56 (B159), CD94 (HP-3D9), CD161 (DX12), NKG2D (1D11), CCR7 (3D12), granzyme B (GB11), and IFN-γ (25723.1) Abs were purchased from BD Biosciences. Anti-CD2 (T11), CD4 (13B8.2), CD7 (8H8.1), CD11a (25.3), CD11b (Bear1), CD25 (B1.49.9), CD27 (1A4CD27), CD33 (D3HL60.251), CD57 (NC1), CD62L (DREG56), CD117 (YB5.B8), CD122 (CF1), CD158a (EB6), and CD158b (GL183) Abs were purchased from Beckman Coulter. Anti-CD34 and CD133 Abs were purchased form Miltenyi Biotec. RIK-2, anti-TRAIL mAb, was prepared as described previously (21).

Isolation of CD34$^+$ and CD133$^+$ cells

Human CB samples were collected from normal full-term deliveries. The parents of all donors provided written informed consent to participate in the study. The procedures were approved by the institutional review board. Mononuclear cells were separated from blood samples by density gradient centrifugation (Lymphoprep; AXIS-SHIELD PoC). CD34- and CD133-enriched cells were separated from mononuclear cells using a MACS Direct CD34 Progenitor Cell Isolation Kit and MACS CD133 MicroBead Kit (Miltenyi Biotec), respectively, according to the manufacturer's protocol. The purity of the CD34$^+$ and CD133$^+$ cells was 97.3 ± 2.3% (n = 15) and 95.4 ± 3.2% (n = 4), respectively. Residual CD3$^+$ and CD56$^+$ cells were 0.73 ± 0.42% and 0.41 ± 0.32%, respectively, in either purification strategy.

Cell culture

Nontissue culture-type 24-well plates were precoated by applying 10 μg/ml Delta4-Fc or control Fc fragments of human Ig G (Fc) (Athens Research & Technology) to the plates at 37°C for 1 h. Cells were cultured in MEM Eagle, α modification (Sigma-Aldrich) supplemented with 20% FBS (Thermo Trace) and penicillin-streptomycin at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. The number of CD34 or CD133 magnetic bead-sorted cells seeded in each well was 0.25–1.2 × 10^5. Cytokines were added at concentrations of 10 ng/ml for IL-7, 100 ng/ml for stem cell factor and 100 ng/ml for FL. one-half of the culture medium was changed every 3 or 4 days. Ten nanograms of thrombopoietin per ml and 100 ng/ml FP6 were added only into the starting culture medium for effective proliferation, although they were not essential (data not shown). IL-15 was added at 5 ng/ml when indicated. Anti-IL-15 or isotype IgG was added at 10 μg/ml when indicated. To inhibit Notch signaling, 10 μmol/L γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *tert*-butyl ester (DAPT; Calbiochem) was added to the culture medium. CD161$^+$ and CD161$^-$ cells from the culture were isolated using FACSAria (BD Biosciences) after staining with anti-CD161-PE Ab.

Phenotyping assay

Immunofluorescence staining for flow cytometry was performed according to standard procedures. To exclude dead cells from the analysis, 7-aminoactinomycin D (Beckman Coulter) was used. Cytoplasmic staining was performed as follows: after staining the cells with anti-CD56-allophycocyanin and fixing with FACS lysing solution (BD Biosciences), the cells were permeabilized using FACS permeabilizing solution (BD Biosciences) and stained with anti-CD3-PE Ab. For staining for granzyme B, the same fixing and permeabilizing procedure was performed after cell surface staining with anti-CD56-PE and anti-CD3-allophycocyanin. For staining for TRAIL, the cells were incubated with 1 μg of RIK-2 for 30 min at 4°C followed by anti-mouse IgG1-PE (A85-1). Cells were analyzed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences).

Cytotoxicity assays

A ^{51}Cr release assay to determine cytotoxicity was performed using standard procedures. In brief, 5 × 10^3 K562 or Jurkat cells were labeled with $Na_2{}^{51}CrO_3$ (Amersham Biosciences) and cocultured with effector cells at various ratios in 96-well round-bottom microtiter plates in 200 μl of culture medium. The cocultured cells were incubated for 4 h, and 100 μl of supernatant was collected from each well and counted with a Packard COBRA gamma counter (Packard Instruments). The percentage of specific ^{51}Cr release was calculated as follows: [cpm experimental release − cpm spontaneous release]/(cpm maximal release − cpm spontaneous release) × 100. The ratio of spontaneous release to maximal release was <20% in all experiments. In experiments to test the mode of cytotoxicity, we used concanamycin A (CMA; Sigma-Aldrich) as a selective inhibitor of the perforin-mediated cytotoxicity, and anti-TRAIL Ab RIK-2. Effectors were pretreated with 100 nmol/L CMA for 2 h before the cytotoxicity assays (22). RIK-2 was added at a final concentration of 10 μg/ml at the start of the cytotoxicity assay.

Intracellular cytokines

The cells were stimulated by PMA (25 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) in the presence of monensin (2 μmol/L; Sigma-Aldrich) for 4 h. After staining the cells with anti-CD56-PE, they were fixed and permeabilized as described above and stained with anti-IFN-γ-FITC Ab. The cells were analyzed on a FACSCalibur using CellQuest software.

Anti-Notch Abs

For cell surface staining, we used biotinylated Abs and streptavidin-PE (BD Biosciences). To block Notch1, we added 10 (μg/ml) MHN1-519 to the medium. Mouse IgG1 (R&D Systems) was used as the control. The anti-human Notch1 (MHN1-519, mouse IgG1), Notch2 (MHN2-25, mouse IgG2a), and Notch3 (MHN3–21, mouse IgG1) mAbs were generated by immunizing BALB/c mice with human Notch1-Fc (R&D Systems), Notch2-Fc (the Fc portion of human IgG1 was fused to the 22nd epidermal growth factor repeat of the extracellular region of human Notch2), or Notch3-Fc (R&D Systems) and screening hybridomas producing mAbs specific for Notch1-Fc, Notch2-Fc, or Notch3-Fc by ELISA. MHN1-519, MHN2-25, and MHN3-21 reacted with CHO(r) cells (23) expressing human Notch1, Notch2, and Notch3, respectively, as demonstrated by flow cytometry (supplemental Fig. S4*A*[10]). MHN1-519 and MHN3h21 blocked Notch1-Fc and Notch3-Fc binding to CHO(r) cells expressing human Delta4, respectively, but MHN2-25 did not block Notch2-Fc binding (supplemental Fig. S4*B*).

[10] The online version of this article contains supplemental material.

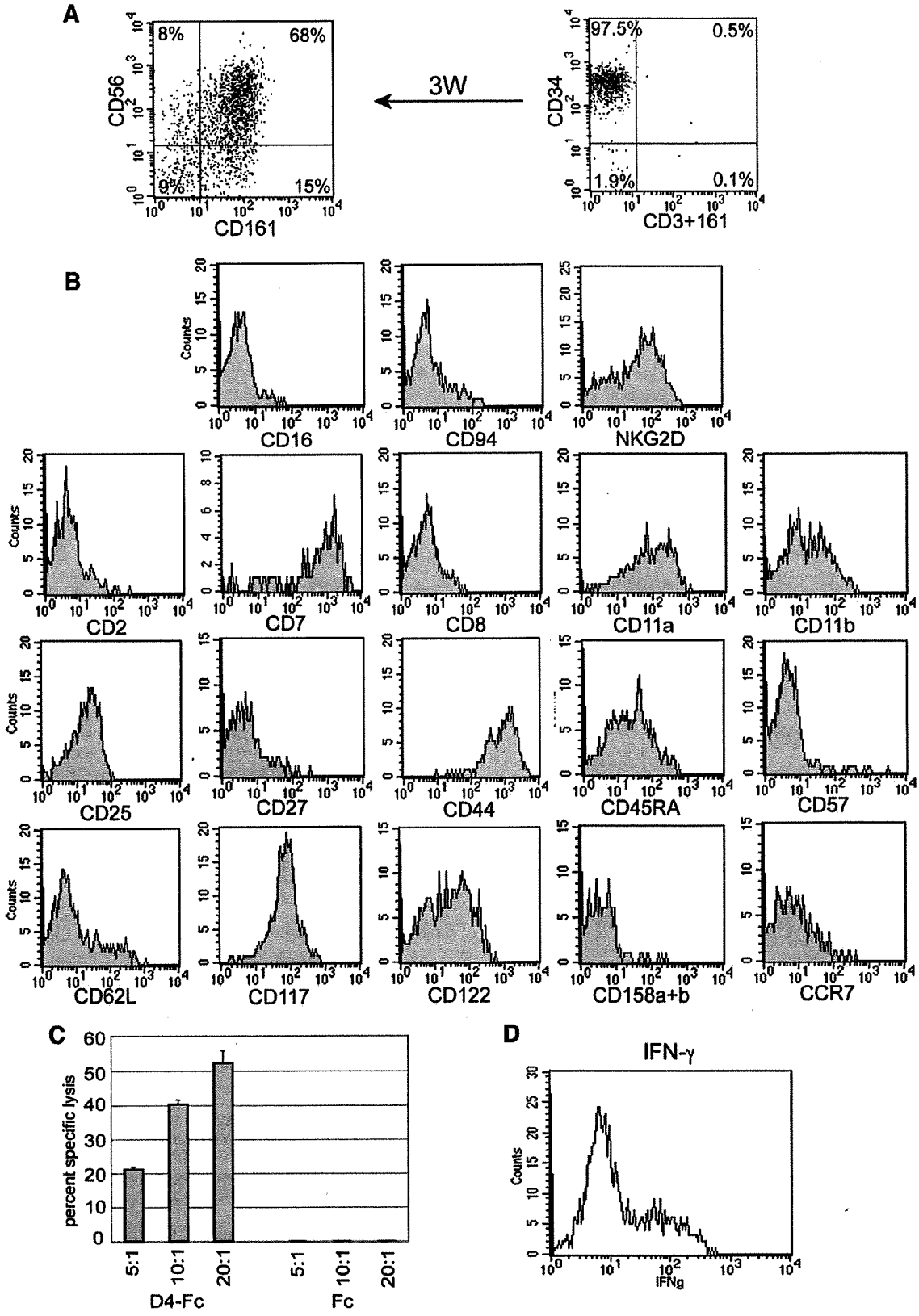

FIGURE 1. Phenotypic and functional analysis of cells derived from $CD34^+$ cells on Delta4-Fc-coated plates. *A*, Representative dot plot illustrating CD161 vs CD56 expression in the cells generated on Delta4-Fc-coated plates from $CD34^+$ CB cells after culture for 3 wk, and dot plot illustrating CD161/CD3 vs CD34 of the sorted CB population before culture. *B*, Various phenotypic analyses of the 3-wk cultured cells that were gated on $CD161^+$ events. Results are representative of at least four experiments. *C*, The 2.5-wk cultured cells were cytotoxic against K562 target cells at the indicated E:T ratios. The ratio of $CD161^+$ cells cultured on Delta4-Fc-coated plates and those Fc-coated plates in this experiment was 40 and 0%, respectively. Results are representative of four experiments. *D*, IFN-γ production by the 3-wk culture cells, as analyzed by intracellular expression. The histogram plots were gated on $CD56^+$events. Results are representative of five experiments.

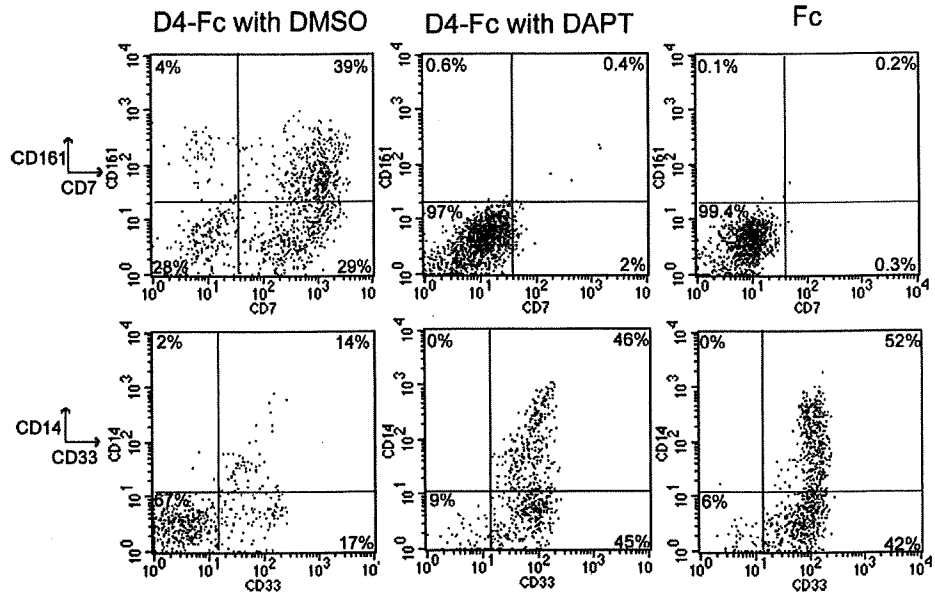

FIGURE 2. Phenotypic analysis of cells cultured in the presence of γ-secretase inhibitors. Representative dot plots of CB $CD34^+$ cells that were cultured for 2.5 wk on Delta4-Fc-coated plates with DMSO (the solvent for the γ-secretase inhibitors: D4-Fc with DMSO), Delta4-Fc-coated plates with DAPT (D4-Fc with DAPT), and Fc-coated plates (Fc). Results are representative of three experiments.

Results

Human CB $CD34^+$ and $CD133^+$ cells gave rise to functional NK cells by Notch signaling in a stroma-free culture without exogenous IL-15

$CD34^+$ or $CD133^+$ cells were cultured on Delta4-Fc-coated plates. The cells became almost immunophenotypically homogeneous after culture for ~3 wk (Fig. 1*A*). The proliferation efficiency depended on CB batches; fold increases in the cell number after the 3-wk culture were 10.3 ± 7.74-fold (n = 11). These cells expressed CD56 and CD161, but did not express surface CD3 or TCRα/β (data not shown). CD56/CD161 double-positive cells also expressed NKG2D and CD117, but were essentially negative for CD16 and killer Ig-like receptors (CD158a and CD158b). The cells had cytotoxic activity against K562 (Fig. 1*C*) and Jurkat cells (see Fig. 5*Bii*), and secreted IFN-γ (Fig. 1*D*). These results indicate that the culture products meet the general criteria for functional NK cells. The products generated from CB $CD34^+$ and $CD133^+$ had the same characteristics (data not shown).

Virtually no NK cells developed in culture on control Fc-coated plates; the vast majority of the cells were $CD33^+$ myeloid cells, a significant part of which expressed CD14 (Fig. 2). The absolute cell numbers with control Fc are ~5-fold higher than that with Delta4-Fc, and the fold increases in the cell number after the 3-wk culture were 45.7 ± 31.6-fold (n = 11). To confirm that the NK cell differentiation was Notch dependent, we added a γ-secretase inhibitor, DAPT, which strongly inhibits ligand-dependent Notch activation (24, 25). The cells cultured on Delta4-Fc-coated plates in the presence of DAPT had the same immunophenotype as those cultured on the control Fc-coated plates and did not give rise to NK cells (Fig. 2), indicating that the observed NK cell development was Notch activation dependent. The number of cells generated increased to the level of that in the control Fc protein-coated plates (data not shown).

We cultured $CD34^+$ cells and $CD133^+$ cells purified from G-CSF-mobilized peripheral blood cells. Both cell types gave rise to $CD56^+CD161^+$ NK cells that were similar to those derived from CB $CD34^+$ or $CD133^+$ cells. The amount of time required for mobilized peripheral blood $CD34^+$ or $CD133^+$ cells (~5 wk) to develop to a major population of $CD56^+CD161^+$ NK cells was greater than that required for CB $CD34^+$ or $CD133^+$ cells (supplemental Figs. S1*A* and S2 and Fig. 3), although the time courses varied to some degree from batch to batch (supplemental Fig. S2 and data not shown).

We next examined the effects of other soluble Notch ligands, human Delta1-Fc and Jagged1-Fc, on NK cell development from CB $CD34^+$ cells. Delta1-Fc had an effect similar to that of Delta4-Fc, although with lower efficiency (supplemental Fig. S1*B*), and Jagged1-Fc showed no potential to induce NK cell development (data not shown). Therefore, we used Delta4-Fc as the soluble Notch ligand and CB $CD34^+$ cells as the starting material for the remaining experiments.

IL-15 is dispensable for in vitro NK cell development from CB $CD34^+$ cells in the presence of Delta4 stimulation, whereas Notch stimulation appears to be essential for physiologic NK cell development

When IL-15 was added to the culture medium on control Fc-coated plates, $CD56^+CD161^+$ NK cells emerged (Fig. 3 and supplemental Fig.S2, Fc plus IL-15; cf with Fig. 3 and supplemental Fig.S2, Fc); this effect was blocked by anti-IL-15- neutralizing Ab (Fig. 3 and supplemental Fig.S2, Fc plus IL-15 plus anti-IL-15). IL-15 does not affect the absolute cell number; fold increases in the cell number after the 3-wk culture were 46.8 ± 36.3-fold, 43.1 ± 35.7-fold, and 48.4 ± 9.48-fold with IL-15 (n = 7), without IL-15 (n = 7), and with IL-15 and anti-IL-15 (n = 3) in the control Fc-coated plate condition. The rate of NK cell development by IL-15 stimulation, however, was much slower than that by Delta4-Fc stimulation. In the absence of Notch stimulation, but with IL-15, the percentage of total NK-lineage cells represented by positive CD161 was only 2.6 ± 2.9%, 6.3 ± 4.6%, and 9.0 ± 4.5% at 2, 3, and 4 wk, respectively (Fig. 3 and supplemental Fig.S2, Fc plus IL-15); whereas in Delta4-Fc with IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc plus IL-15) or without IL-15 (Fig. 3 and supplemental Fig.S2, D4-Fc), the percentage of total NK-lineage cells was 56 ± 17%, 77 ± 11%, and 81 ± 5.8% (with IL-15) or 52 ± 18%, 74 ±