Combined immunodeficiency with CD4 lymphopenia and sclerosing cholangitis caused by a novel loss-of-function mutation affecting IL21R

Combined immunodeficiencies (CIDs) comprise a heterogeneous group of monogenic disorders manifesting with lymphocyte defects, recurrent infections and dysregulated immune response. Recently, we and others have described clinical and molecular features of the combined immunodeficiency syndromes caused by *IL21* or *IL21R* loss-of-function mutations in humans. To date, only one homozygous mutation in the *IL21* and five distinct homozygous mutations in the *IL21R* gene were identified, however only three have been published so far.[1,2] Accordingly, comprehensive clinical and immunological data characterizing this novel type of CID are lacking. Here we report a novel homozygous frame-shift mutation in the *IL21R* gene of a patient presenting with T-, B- and natural killer (NK)-cell lymphopenia. The patient had eosinophilic duodenitis, sclerosing cholangitis, tinea corporis and otitis media, but no increased frequency of respiratory infections, as described in other IL21R-deficient patients.[3] Thus, our report expands on the clinical and functional characteristics of the IL21R deficiency, which may aid early diagnosis leading to improved treatment options.

The cytokine IL21 regulates T- and B-cell proliferation and activation,[4,5] and promotes NK-cell cytotoxicity.[6] IL21 binds to the IL21R, primarily expressed by lymphocyte populations and other hematopoietic cells, but it can also be found on non-hematopoietic cells including fibroblasts, keratinocytes and intestinal epithelial cells.[7,8] IL21R and the interleukin-2 receptor common gamma-chain (IL2RG) form a heterodimer complex which upon IL21 binding initiates downstream signaling by activating Janus kinase 1, 3 (JAK1, JAK3) and inducing signal transducer and activator of transcription 1, 3 and 5 (STAT1, STAT3, STAT5) phosphorylation.[9] In affected individuals, loss-of-function mutations in the *IL21R* gene led to defective B-cell differentiation, impaired T-cell cytokine production, and impaired NK-cell cytotoxicity. Clinically these patients were characterized by cryptosporidiosis associated with severe chronic cholangitis leading to liver failure and respiratory tract infections, typically seen in patients with CID.[2] Recently, another IL21R-deficient patient suffering from chronic respiratory tract infections, however without cryptosporidium-associated cholangitis, was identified.[2] We recently described an IL21-deficient patient who was also cryptosporidium-negative and had no signs of cholangitis, but presented with very early-onset inflammatory bowel disease (IBD) which masked underlying CID at early age.[1] Thus the diversity of clinical phenotypes of the IL21 signaling defects prompts for identification and analysis of further patients to ultimately improve therapy.

Our patient is a 7-year old Turkish boy born to healthy first-degree consanguineous parents (*Online Supplementary Figure S1A*). His older sister, who had chronic diarrhea and abdominal distention, died at the age of five. The patient's complaint of chronic diarrhea started at the age of six months. Endoscopic biopsy revealed villous atrophy and blunting in focal areas, minimal increase in intraepithelial lymphocytes, eosinophilic leukocyte infiltration in lamina propria, minimal edema and inflammation in stroma leading to the diagnosis of eosinophilic duodenitis.

The patient was referred to our immunology department for an investigation of potential underlying immunodeficiency at four years of age. In addition to chronic diarrhea, he suffered from recurrent otitis media, tinea corporis, tooth abscesses and recurrent herpes labialis. Physical examination revealed failure to thrive [weight 11 kg (2.3 kg <3 percentile); height 84 cm (12 cm <3 percentile)], hepatomegaly (7 cm below the costal margin) and a perforated left tympanic membrane. Endocrinological findings

Table 1. Laboratory findings of the IL21R-deficient patient.

	Patient's values						
Immunoglobulins							
Test date	**03.2010**	**10.2010**	**02.2011**	**09.2011**	**12.2011**	**03.2012**	**12.2013**
IgA (mg/dL)	92 (44-244)	79 (44-244	60 (44-244	60 (57-282)	122 (44-244)	139 (44-244)	71 (44-244)
IgG (mg/dL)	711 (640-2010)	810* (640-2010)	750* (640-2010)	750* (745-1804)	950* (745-1804)	1060* (745-1804)	**668*** (745-1804)
IgM(mg/dL	144 (52-297)	92 (52-297)	54 (52-297)	84 (78-261)	86 (78-261)	78 (78-261)	98 (78-261)
IgE (IU/mL)	716						
Lymphocyte subsets							
Test date	**03.2010**	**08.2010**	**10.2010**	**08.2012**	**11.2013**	**12.2013**	
CD3 (%)	66 (56-75)	60 (56-75)	72 (56-75)	80 (56-75)	79 (56-75)	81 (56-75)	
CD4 (%)	**15** (28-47)	**20** (28-47)	**10** (28-47)	**19** (28-47)	**22** (28-47)	**22** (28-47)	
CD8 (%)	**40** (16-30)	**46** (16-30)	**44** (16-30)	**48** (16-30)	**43** (16-30)	**47** (16-30)	
CD16/56 (%)	**1** (4-17)	**0** (4-17)	**2** (4-17)	14 (4-17)	**1** (4-17)	1 (4-17)	
CD19 (%)	30 (14-33)	**41** (14-33)	28 (14-33)	15 (14-33)	18 (14-33)	15 (14-33)	
Blood counts							
Test date	**03.2010**	**08.2010**	**10.2010**	**08.2012**	**11.2013**	**12.2013**	
WBC (cells/ μL)	9400 (5200-11000)	**19400** (5200-11000)	**9800** (5200-11000)	**13500** (5200-11000)	**14300** (5200-11000)	**15100** (5200-11000)	
ALC (cells/ μL)	**2100** (2300-5400)	**800** (2300-5400)	4600 (2300-5400)	**5800** (2300-5400)	**6500** (2300-5400)	5200 (2300-5400)	
$CD4^+$ T cells	315 (700-2200)	160 (700-2200)	460 (700-2200)	1102 (700-2200)	1430 (700-2200)	1144 (700-2200)	
T cell proliferation							
Stimulus	**PHA**	**ConA**	**PMA+Iono**				
Patient (cpm)	**3428**	**5764**	**2721**				
Healthy control (cpm)	41355	32437	25513				

*Values in the brackets show reference ranges[19,20]. PHA: phytohemagglutinin; ConA: concanavalin-A; PMA: phorbol-12-myristate-13-acetate; Iono: ionomycin. *Values obtained during immunoglobulin treatment. Abnormal values are printed in bold.*

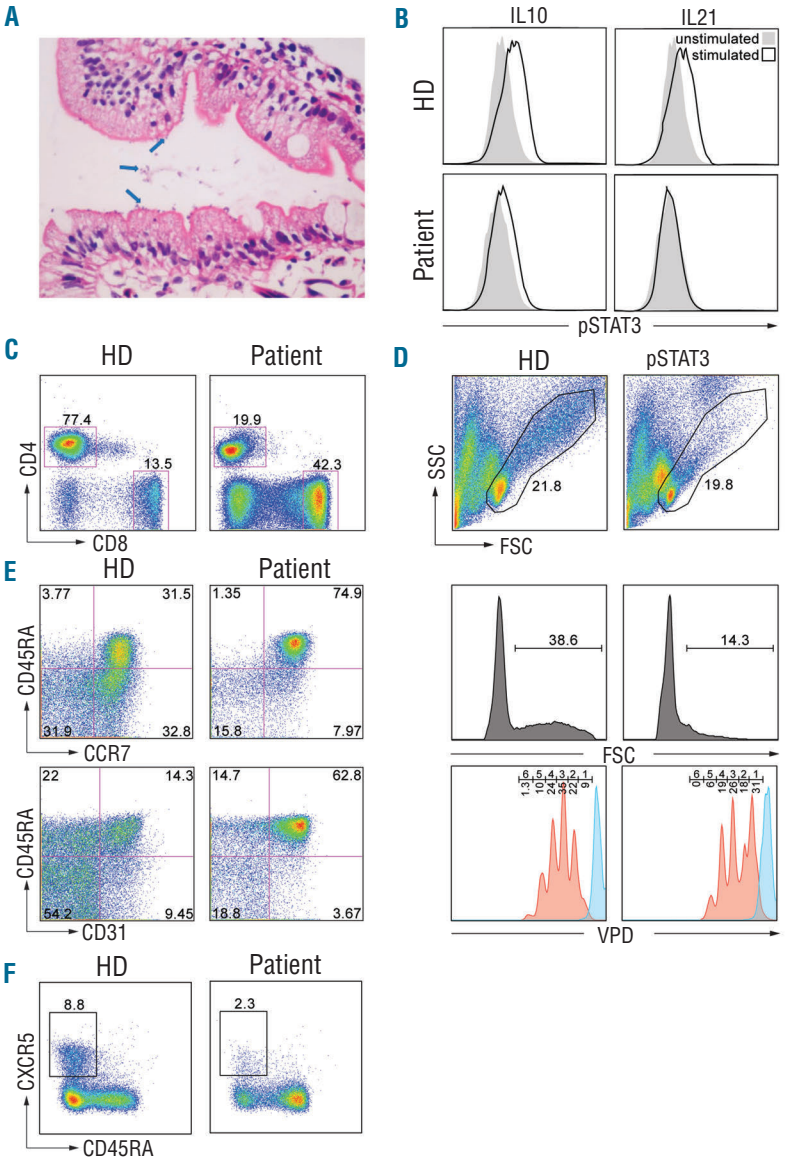

Figure 1. Clinical, immunological and functional phenotype of the IL21R-deficient patient. (A) Hematoxylin & Eosin staining of the duodenal biopsy displaying cyptosporidium parasites attached to the epithelial surface. (B) Intracellular flow cytometry analysis of the STAT3 phosphorylation in $CD4^+$ T cells after stimulation with IL10 (50 ng/ml) and IL21 (10 ng/mL), respectively. (C) Flow cytometry analysis of $CD4^+$ and $CD8^+$ T-cell populations in PBMCs. (D) Proliferation analysis of PBMCs four days after stimulation with anti-CD3 and anti-CD28 antibodies detected by monitoring dilution of the violet proliferation dye (VPD). The percentages of cells in the respective gates are indicated in the plots. Bottom panel: the numbers above the indicated gates refer to the number of divisions. (E) Analysis of naïve and memory (top), and $CD31^+CD45RA^+$ recent thymic emigrants (bottom) within the $CD4^+$ T-cell population. (F) Analysis of $CXCR5^+CD45RA^-$ T_{FH} cells from the IL21R-deficient patient and a healthy adult control. HD: healthy adult donor.

revealed reduced IGF-1 growth hormone levels (5.95 ng/mL; normal range 23.9-392), possibly due to hepatomegaly and malnutrition. Basic immunological workup showed normal B- and T-cell counts, while natural killer (NK) cells were almost absent (Table 1). Moreover, subset-specific analysis revealed inverted CD4/CD8 ratio, characterized by intermittent marked decrease of CD4 T cells (Table 1). T-cell proliferation upon stimulation with phytohemagglutinin (PHA), concanavalin-A (ConA) or phorbol-12-myristate-13-acetate combined with ionomycin (PMA/Iono) was significantly impaired (Table 1). Trimethoprim/sulfamethoxazole prophylaxis and intravenous immunoglobulin replacement therapy was started.

During follow up, the patient had elevated liver enzymes (ALT 389 U/L, AST 313 U/L, GGT 255 U/L, ALP 1275 U/L), while vitamin A, E, and D levels decreased. Liver biopsy revealed sclerosing cholangitis. Ursodeoxycholic acid, kreon, and lipid soluble vitamin supplementation was commenced. During hospitalization for a severe episode of diarrhea, histopathological analysis revealed presence of cryptosporidium in stool and at the apical surface of the duodenal epithelium (Figure 1A), which was treated with azithromycin and continued as prophylactic therapy (10 mg/kg/day). The patient was negative for cryptosporidium in stool by recent microscopic analyses. Serum cytomegalovirus PCR test was negative.

To identify the underlying genetic disease etiology, we employed custom-designed targeted enrichment followed by massively parallel sequencing of 356 genes implicated in immune functions including 248 known primary immunodeficiency (PID) genes[10] and additional PID genes recently published or presented at conferences at the time of the gene panel design. While no mutations in CD4 T-cell lymphopenia associated genes such as *CD40, CD40L, CIITA, RFX5, RFXAP, RFXANK, ITK, MST1* or *LCK* were identified, we found a novel homozygous frame-shift mutation

in *IL21R* (c.535delG) causing a premature stop codon instead of Ser229, 51 amino acids downstream of the mutation (p.Asp179Thrfs*51) (*Online Supplementary Figure S1B*). The mutation was validated by capillary sequencing and showed segregation consistent with an autosomal recessive mode of inheritance (*Online Supplementary Figure S1A*).

A number of cytokine/receptor systems including IL21/IL21R and IL10/IL10R signal via JAK/STAT pathway.[11] IL-21R signaling induces expression of genes which promote GC formation, class switch recombination (CSR) and plasma cell differentiation[4] by STAT3-independent[12] and STAT3-dependent[13] mechanisms. The frame-shift mutation in the *IL21R* gene of the index patient correlated with the loss of STAT3 phosphorylation in lymphocytes after stimulation with IL21, which was readily detectable after IL10 stimulation (Figure 1B and *data not shown*), thus confirming loss of IL21R function. In contrast to previously reported patients,[3] immunophenotyping of the index patient revealed a markedly reduced relative proportion of CD4 T cells (Figure 1C). Since the PHA, PMA/Iono or concanavalin A-stimulated T-cell proliferation was significantly impaired similar to previously reported patients,[3] we analyzed this proliferation defect on a level of $CD4^+$ and $CD8^+$ T-cell subsets by measuring cell divisions of violet proliferation dye-labeled PBMCs after T-cell receptor (TCR) stimulation. Upon *in vitro* anti-CD3/anti-CD28 stimulation, the relative number of blasting cells was lower compared to a healthy control (Figure 1D, top and middle) and was accompanied by the reduced upregulation of the activation markers CD69 and CD25 (*Online Supplementary Figure S1C*). However, the responding cells proliferated at levels comparable to a healthy control (Figure 1D, bottom) suggesting a developmental defect leading to reduced numbers of T cells able to respond to TCR stimulation. Accordingly, the majority of patient's CD4 T cells were naïve $CD45RA^+CD31^+CCR7^+$ recent thymic emigrants, while the $CD45RA^-$ memory populations and follicular helper T cells (T_{FH}) were strongly reduced (Figure 1E and F) pointing towards defects in the germinal center-dependent generation of memory B cells, as initially observed in IL21R-deficient patients.[3] Interestingly, although the $CD8^+CD45RA^-$ memory T-cell populations were not reduced (*Online Supplementary Figure S1D*), CD8 T cells also responded to the TCR stimulation at reduced frequency. Very few $CD27^+IgD^-$ memory B cells could be detected in the index patient (Figure 2A), consistent with the central role of IL21 signaling in this process.[12] Moreover, an increased relative proportion of $CD10^+CD38^+$ transitional B cells was observed (Figure 2B and *Online Supplementary Figure S1E*). Although this was not noted in the previously reported IL21R-deficient patients, we observed similar developmental defects in the IL21-deficient patient,[1] pointing towards the role of IL21 signaling in the maturation of peripheral B cells. Interestingly, predominantly normal serum immunoglobulin levels in the patient (Table 1) suggested sufficiency of IL21R-independent pathways for plasma cell differentiation despite near absence of memory B cells. One exception was elevated serum IgE levels consistent with the inhibitory role of IL21 in germ-line Cε transcription.[14-16] One important finding that extends the spectrum of phenotypes of the IL21R-deficient patients is profoundly reduced $CD56^+$ NK cells, which in addition displayed a predominantly immature $CD57^-$ phenotype (Figure 2C), suggesting functional NK-cell defects. Although IBD-like inflammation observed in the index patient may be caused by the cryptosporidiosis, it may also be a consequence of functional dysregulation of the lymphoid system. Recently, NK cells

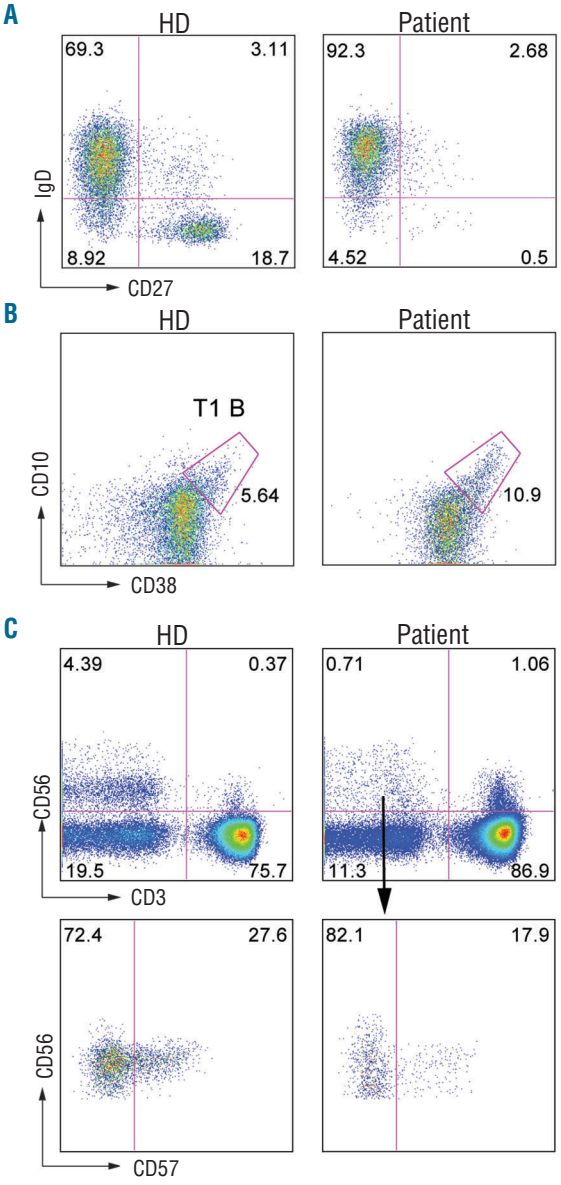

Figure 2. Phenotypic analysis of IL21R-deficicent B- and NK-cell populations. (A) Flow cytometry analysis of patient's and healthy adult donor's naïve and memory B-cell populations gated on $CD19^+$ B cells. The percentages of cells in the respective quadrants are indicated. (B) Analysis of immature transitional B cells from the patient and a healthy adult control defined by the surface expression of CD10 and CD38 after gating on $CD19^+$ B cells. (C) Identification (top) and maturation analysis (bottom) of $CD56^+CD3^-$ NK cells from the patient and a healthy adult control. The percentages of cells in the respective quadrants are indicated.

were shown to down-regulate pro-inflammatory functions of neutrophils thereby protecting against colitis in a mouse model.[17] Thus reduced NK-cell numbers in the index patient could contribute to the eosinophilic duodenitis, possibly caused by cryptosporidiosis.

Two previously reported cases of IL21R deficiency underwent an HLA-identical hematopoietic stem cell transplant (HSCT), but the post-transplant course was complicated by an escalation of cholangitis and cytomegalovirus infections leading to death from multi-organ failure. This suggested that early diagnosis and immediate HSCT may

be critically important for the curative outcome.[3] However, since cryptosporidium infection-associated severe sclerosing cholangitis adversely affected outcome of the HSCT in number of CD40L-deficient patients,[18] there are no clear guidelines as to whether allogeneic HSCT should be recommended to cryptosporidium-infected IL21R-deficient patients, and more comprehensive studies in a larger cohort of patients will be urgently needed.

In conclusion, we here identify a novel biallelic loss-of-function mutation affecting the *IL21R* gene in a patient with combined immunodeficiency. In contrast to previous studies, the patient had marked CD4- and NK-lymphopenia. Thus, the phenotype of IL21(receptor) deficiency may be considerably more diverse than previously appreciated, and molecular analysis of patients with unclear genetic etiology of combined immunodeficiency should include the *IL21(receptor)* genes.

Baran Erman,[1,2] Ivan Bilic,[1*] Tatjana Hirschmugl,[1] Elisabeth Salzer,[1] Deniz Çagdas[2] Saliha Esenboga,[2] Zuhal Akcoren,[3] Ozden Sanal,[2] Ilhan Tezcan,[2*] and Kaan Boztug[1,4*]*

[1]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria; [2]Department of Pediatric Immunology, Hacettepe University of Medicine, Ankara, Turkey; [3]Department of Pediatric Pathology, Hacettepe University Faculty of Medicine, Ankara, Turkey; and [4]Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Austria

**BE and IB, as well as IT and KB, contributed equally to this work.*

Acknowledgments: the authors would like to thank the patient and his family for participating in the study. The help of all contributing medical, technical and administrative staff is greatly acknowledged. The authors thank Cecilia Domínguez Conde and Katharina Willmann for critical reading of the manuscript and scientific discussions.

Funding: supported by funding from the Austrian Science Fund (FWF) grant number P24999 awarded to Kaan Boztug.

Correspondence: kboztug@cemm.oeaw.ac.at/ fetezcan@hacettepe.edu.tr

doi:10.3324/haematol.2014.120980

Key words: combined immunodeficiencies, loss-of-function mutation, IL21R, CD4 lymphopenia, sclerosing cholangitis.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Salzer E, Kansu A, Sic H, et al. Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency. J Allergy Clinical Immunol. 2014;133(6):1651-1659 e12.
2. Kotlarz D, Zietara N, Milner JD, Klein C. Human IL-21 and IL-21R deficiencies: two novel entities of primary immunodeficiency. Curr Opin Pediatr. 2014;26(6):704-712.
3. Kotlarz D, Zietara N, Uzel G, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med. 2013;210(3):433-443.
4. Parrish-Novak J, Foster DC, Holly RD, Clegg CH. Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. J Leukoc Biol. 2002;72(5):856-863.
5. Ettinger R, Sims GP, Fairhurst AM, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. J Immunol. 2005;175(12):7867-7879.
6. Kasaian MT, Whitters MJ, Carter LL, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. Immunity. 2002;16(4):559-569.
7. Distler JH, Jungel A, Kowal-Bielecka O, et al. Expression of interleukin-21 receptor in epidermis from patients with systemic sclerosis. Arthritis Rheum. 2005;52(3):856-864.
8. Monteleone G, Caruso R, Fina D, et al. Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. Gut. 2006;55(12):1774-1780.
9. Spolski R, Leonard WJ. Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu Rev Immunol. 2008;26:57-79.
10. Keerthikumar S, Raju R, Kandasamy K, et al. RAPID: Resource of Asian Primary Immunodeficiency Diseases. Nucleic Acids Res. 2009;37(Database issue):D863-867.
11. Kane A, Deenick EK, Ma CS, Cook MC, Uzel G, Tangye SG. Curr Opin Immunol. 2014;28:49-57.
12. Avery DT, Deenick EK, Ma CS, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. J Exp Med. 2010;207(1):155-171.
13. Deenick EK, Avery DT, Chan A, et al. Naive and memory human B cells have distinct requirements for STAT3 activation to differentiate into antibody-secreting plasma cells. J Exp Med. 2013;210(12):2739-2753.
14. Suto A, Nakajima H, Hirose K, et al. Interleukin 21 prevents antigen-induced IgE production by inhibiting germ line C(epsilon) transcription of IL-4-stimulated B cells. Blood. 2002;100(13):4565-4573.
15. Ozaki K, Spolski R, Feng CG, et al. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002;298(5598):1630-1634.
16. Zotos D, Coquet JM, Zhang Y, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J Exp Med. 2010;207(2):365-378.
17. Hall LJ, Murphy CT, Quinlan A, et al. Natural killer cells protect mice from DSS-induced colitis by regulating neutrophil function via the NKG2A receptor. Mucosal Immunol. 2013;6(5):1016-1026.
18. Gennery AR, Khawaja K, Veys P, et al. Treatment of CD40 ligand deficiency by hematopoietic stem cell transplantation: a survey of the European experience, 1993-2002. Blood. 2004;103(3):1152-1157.
19. Shearer WT, Rosenblatt HM, Gelman RS, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003;112(5):973-980.
20. Tezcan I BA, Ersoy F, Sanal O. Saglıklı Türk çocukları ve erişkinlerde turbidimetrik yöntemle bakılan serum immunoglobulin düzeyleri. Çocuk Saglıgı ve Hastalıkları Dergisi. 1996(39):649-56.