3. Results

3.1. Thalidomide does not block caspase-1 activation or IL-1β production following LPS stimulation in CGD monocytes

Monocytes are capable of activating caspase-1 to process and release IL-1β by LPS signaling through Toll-like receptor 4 (TLR4) [11]. In keeping with these results [12], LPS induced capase-1 activation and IL-1β production in a dose-dependent manner in CGD monocytes as well as in healthy control monocytes (Figs. 1a–c), suggesting that ROS generated by NADPH oxidase was dispensable for caspase-1 activation and conversion of pro-IL-1β to IL-1β. To assess the effect of thalidomide on caspase-1 activation and IL-1β production, monocytes were cultured with LPS in the presence of thalidomide and the levels of active caspase-1 in monocytes and IL-1β in culture supernatants were measured. Thalidomide did not show any significant effects on caspase-1 activation and IL-1β production in monocytes from healthy control and CGD patients (Figs. 1d–e). On the other hand, CCCP, a chemical inhibitor of ROS-generating mitochondria [13,14], significantly reduced LPS-driven IL-1β production in CGD monocytes and healthy control monocytes (Fig. 1e). Since CCCP showed no effect on caspase-1 activation (Fig. 1d), it was likely that CCCP inhibited IL-1β production in monocytes stimulated with LPS independent of the inflammasome.

3.2. Lipopolysaccharide initiates inflammatory cytokine production in CGD monocytes treated with thalidomide

LPS signaling via TLR4 induces the production of IL-6, IL-8, and TNF-α, which is required for resistance against pathogenic microorganisms. Whereas maturation of IL-1β depends on inflammasome oligomerization followed by caspase-1 activation, the cytokines are secreted through activation of the NF-κB pathway. CGD monocytes had higher IL-6 and

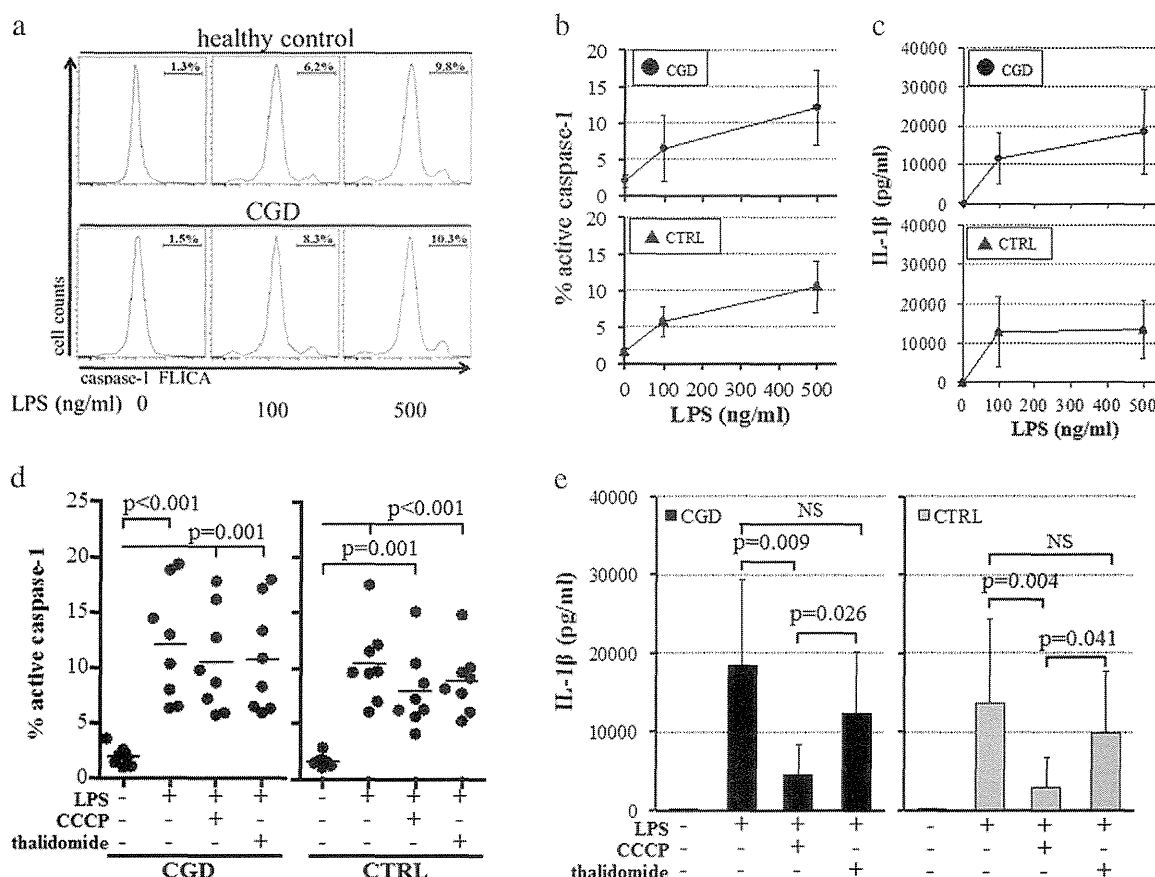

Figure 1 Lipopolysaccharide activates caspase-1 and induces IL-1β secretion in CGD monocytes treated with thalidomide. (a–c) Monocytes were stimulated with 0, 100, and 500 ng/ml lipopolysaccharide (LPS) for 4 h. (a) The numbers above the bracketed lines indicate the percentage of active caspase-1-positive monocytes quantified using FLICA. (b) Active caspase-1 levels were assessed in monocytes from CGD patients (black circles) and healthy controls (CTRL; triangles). (n = 8) (c) LPS-driven IL-1β production was assessed in monocytes from CGD patients (black circles) and healthy controls (triangles). (n = 8) (d) Shown is the percentage of active caspase-1-positive cells in monocytes stimulated with 500 ng/ml LPS alone or LPS plus 50 nM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or 10 μg/ml thalidomide for 4 h. (e) Secretion levels of IL-1β were assessed in monocytes from CGD patients (black bars) and healthy controls (gray bars) after stimulation with 500 ng/ml LPS alone or LPS plus 50 nM CCCP or 10 μg/ml thalidomide for 4 h. (n = 8) NS, not significant.

TNF-α production in response to LPS than that of healthy controls, suggesting that CGD monocytes retained the ability to activate and easily release more inflammatory cytokines at the time of infection (Fig. 2). Moreover, thalidomide did not impair the production of IL-6, IL-8, and TNF-α in monocytes stimulated with LPS both from healthy controls and CGD patients, while CCCP strongly suppressed the LPS-driven production of cytokines similar to IL-1β (Fig. 2). Since production of inflammatory cytokines depends on NF-κB signaling and is less associated with inflammasome oligomerization induced by mitochondria-generating ROS, CCCP may inhibit cytokine production in a manner not susceptible to ROS inhibitors. Again, thalidomide did not interfere with production of inflammatory cytokines by LPS.

3.3. Thalidomide suppresses NF-κB activation induced by TNF-α

Having identified that thalidomide had no effect on the production of cytokines induced by LPS, we next assessed the effect of thalidomide on the signal cascade induced by TNF signaling. When peripheral blood mononuclear cells (PBMCs) were preincubated with thalidomide and then stimulated with TNF-α, thalidomide significantly suppressed the DNA-binding ability of NF-κB in a dose-dependent manner in monocytes from CGD patients and healthy controls (Fig. 3). These results are identical to those previously described, that is, thalidomide suppressed NF-κB activation induced by TNF-α, but did not inhibit the activation by LPS [15]. This suggests that thalidomide can suppress excessive inflammation induced by TNF-α signals associated with granuloma formation in CGD colitis without interfering with LPS-induced inflammatory cytokine production.

3.4. Thalidomide suppresses the secretion of IL-1β induced by ATP in CGD monocytes

More than 95% of pro-IL-1β in monocytes stimulated by LPS is unprocessed and dispersed throughout the cytoplasm absent in a secondary stimulus. Recently, the stimulus is thought to be signaling through pannexin-1, which functions as a hemichannel, induced by the binding of ATP to P2X7 receptors [16,17]. Indeed, application of ATP to human monocytes did not induce a large amount of IL-1β secretion; ATP induced significantly higher levels of IL-1β secretion in LPS-primed monocytes from CGD patients and healthy controls (Fig. 4 compared to Fig. 1e). Interestingly, thalidomide decreased IL-1β secretion from CGD monocytes in a dose-dependent manner (Fig. 4). Although the exact mechanism remains unclear, this suggests that thalidomide inhibits IL-1β secretion enhanced by ATP in CGD monocytes.

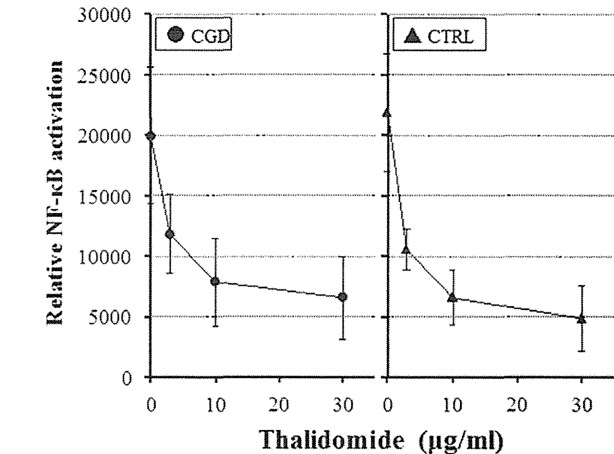

Figure 3 Thalidomide suppresses the TNF-α-mediated DNA-binding ability of NF-κB. Nuclear extracts from peripheral blood mononuclear cells (PBMCs) from CGD patients (circle) and healthy controls (triangle) were assessed for the DNA-binding ability of NF-κB at each indicated concentration of thalidomide. PBMCs were treated with serial concentrations of thalidomide for 2 h and then stimulated with 10 ng/ml TNF-α for 30 min at 37°. NF-κB activation was monitored by luciferase activity. (n = 8).

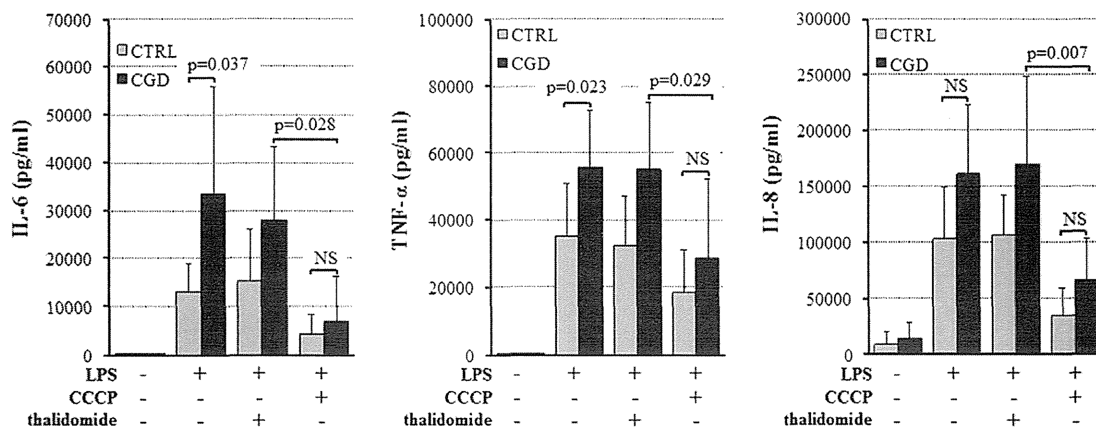

Figure 2 Lipopolysaccharide-driven inflammatory cytokine production was not impaired in CGD monocytes treated with thalidomide. Monocytes were stimulated with 500 ng/ml LPS alone or LPS plus 50 nM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) or 10 μg/ml thalidomide for 4 h. CGD monocytes (black bars) released significantly higher amounts of IL-6 and TNF-α in response to LPS than monocytes from healthy controls (gray bars: CTRL). There was no difference in LPS-driven IL-8 secretion between monocytes from CGD patients and healthy controls (p = 0.053). (n = 8). NS, not significant.

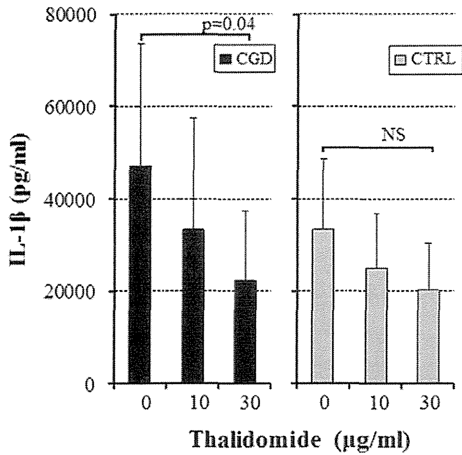

Figure 4 IL-1β secretion induced by ATP in CGD monocytes. After activation by 100 ng/ml LPS alone or LPS plus serial concentrations of thalidomide for 2 h, ATP was added to achieve a final concentration of 5 mM and the samples were incubated at 37° for 4 h. Secretion of IL-1β into culture supernatants was determined in 5 × 10^6 cells/ml monocyte cell cultures from CGD patients (black bars) and healthy controls (gray bars: CTRL). (n = 8) NS, not significant.

3.5. Thalidomide therapy improves clinical symptoms of CGD colitis without impairing control of *Aspergillus* pneumonia and BCG lymphadenitis present at the start of thalidomide therapy

A patient with intractable CGD colitis was treated with 1.5 mg/kg of thalidomide, while also taking 0.2 mg/kg oral corticosteroid and 1.5 mg/kg azathioprine. His bowel inflammatory lesions were located in colon and the clinical symptoms of colitis were evaluated using Pediatric Ulcerative Colitis Activity Index (PUCAI) scoring, a validated measurement of ulcerative colitis disease activity in children and adolescents [18]. Since thalidomide therapy was initiated, an improvement was seen in the symptoms of body weight loss and anemia and the disease activity by PUCAI (Figs. 5a and b). In line with his clinical improvements, his serum levels of TNF-α, IL-6, and IL-8 decreased at 6 to 8 weeks of thalidomide therapy (Fig. 5c), while serum IL-1β was undetectable during thalidomide therapy (data not shown). Regarding immune function, the number of $CD3^+$ T cells, $CD19^+$ B cells, and $CD4^+$ $CD45RA^+$ $CD31^+$ T cells that are thought to represent thymopoiesis increased after thalidomide therapy (Fig. 5d). The level of phytohemagglutinin-induced blastoid transformation was 33,300 cpm at 8 weeks of thalidomide treatment, which was within the normal range.

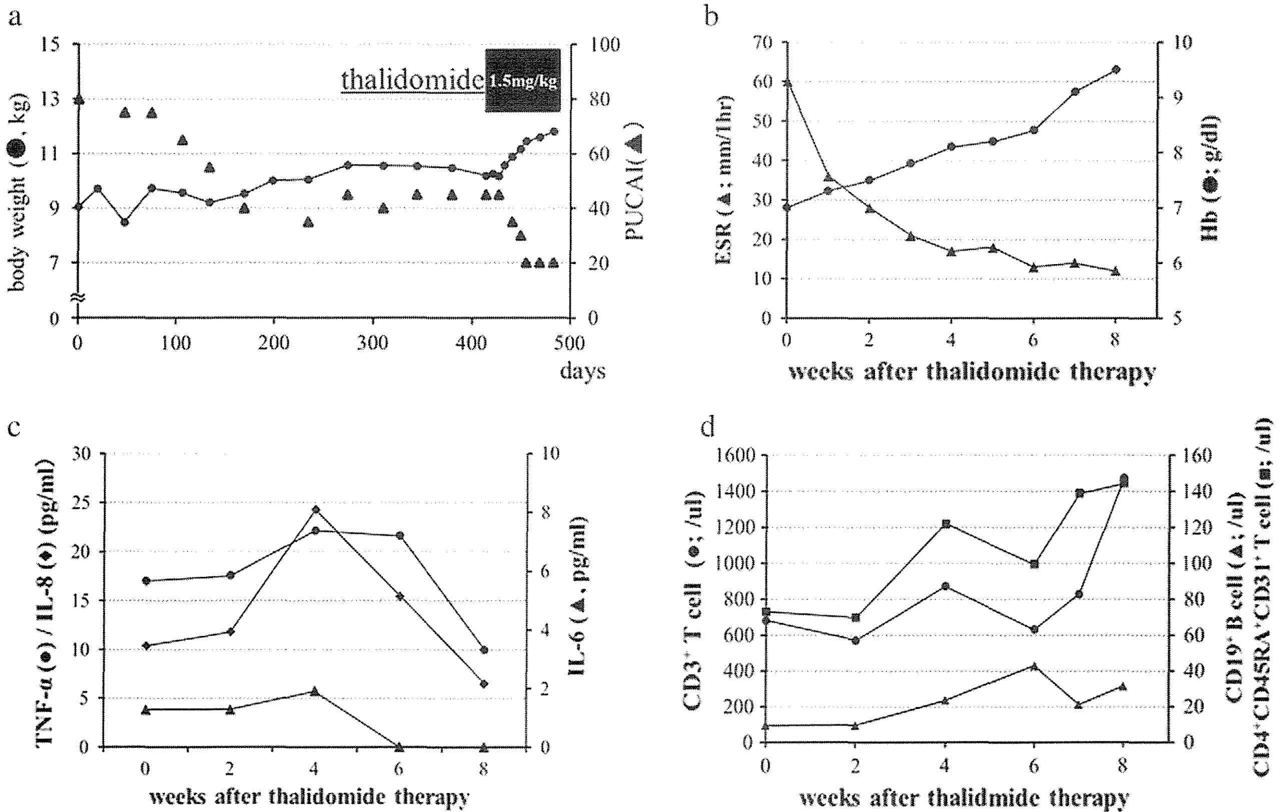

Figure 5 Thalidomide therapy improves symptoms of CGD colitis without immune suppression in a patient with CGD. A patient with CGD colitis received thalidomide therapy for 8 weeks. (a) After initiation of thalidomide therapy at day 421 of corticosteroid therapy, the patient gained 2 kg, and the pediatric ulcerative colitis activity index (PUCAI) improved from 45 to 20. (b) The erythrocyte sedimentation rate (triangles) decreased to 12 mm/h from 60 mm/h, and serum hemoglobin (circles) increased to 9.5 g/dl from 7 g/dl after 8 weeks of thalidomide therapy. (c) Serum levels of TNF-α (circles), IL-6 (triangles), and IL-8 (diamonds) decreased after 4 weeks of thalidomide therapy. (d) The number of $CD3^+$ T cells (circles), $CD4^+$ $CD45RA^+$ $CD31^+$ naïve T cells (squares), and $CD19^+$ B cells (triangles) in peripheral blood was analyzed by flow cytometry following thalidomide therapy.

The patient's pulmonary aspergillosis and *BCG*-related lymphadenitis that developed prior to thalidomide therapy were not aggravated and no other infections were identified during thalidomide therapy. Thalidomide treatment ceased at 8 weeks, and the conditioning for bone marrow transplantation was initiated next. The relapse of CGD colitis was completely eliminated by myeloablative conditioning and bone marrow transplantation after thalidomide therapy.

4. Discussion

The paradoxical clinical manifestations of CGD, susceptibility to infection and excessive inflammation, make it difficult to choose an appropriate course of therapy for patients with CGD colitis that develops through dysregulated inflammatory cytokine production [19–21]. The reason for this may be attributed to our insufficient understanding of a role for ROS on inflammasome activation. CGD patient monocytes were predisposed to secrete IL-1β in response to LPS, while CGD patients inherently lack NADPH oxidase activity in phagocytes. Furthermore, CGD monocytes stimulated with LPS activated caspase-1 comparable to those of healthy controls. These results suggest that ROS generated by NADPH oxidase were dispensable for NLRP3 inflammasome activation, or it seems to be more proper to say that ROS inhibit prolonged hyper-inflammation [22,23]. This hypothesis was also supported by results recently published [12]. They demonstrated that monocytes with p47phox deficiency activated caspase-1 and secreted IL-1β in response to LPS. Moreover, IL-1β secretion by CGD monocytes stimulated by uric acid crystals was 4-fold higher compared with that of healthy controls. Importantly, they showed that the small molecule diphenylene iodonium (DPI), an inhibitor of ROS, inhibited the production of TNF-α as well as IL-1β, and determined that DPI exerted the effects independently of the inflammasome. In the present study, we used the small molecule CCCP as an inhibitor of ROS generated by mitochondria and obtained similar results showing that CCCP inhibited production of inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α despite of no inhibition of caspase-1 activation. Together with the results of DPI, IL-1β production seems to be regulated by some mechanism other than the NLRP3 inflammasome. These underlying mechanisms that result in hyper-inflammation may explain why CGD patients frequently suffer from exaggerated bowel inflammation.

Unexpectedly, thalidomide did not show any inhibitory effects on the production of inflammatory cytokines in monocytes stimulated with LPS, but did inhibit the NF-κB activation when monocytes from CGD patients or healthy controls were stimulated with TNF-α. This curious difference has already been reported previously [15]. Although the mechanism remains unclear, thalidomide is considered to have partly negative effects on NF-κB signaling, showing anti-inflammatory activities [15]. Interestingly, while thalidomide has negative effects on NF-κB activation induced by TNF-α in a dose-dependent manner in hematopoietic and epithelial cells, the inhibitory effects were not observed in cells stimulated with LPS. The selective suppression of NF-κB by thalidomide is considered to be partly due to inhibition of an inhibitory subunit of NF-κB (IkBa), abrogation of IkBa activation, and inhibition of NF-κB-dependent reporter gene expression [15,24]. Furthermore, thalidomide inhibited IL-1β secretion in CGD monocytes that was augmented by addition of ATP in a dose-dependent manner, which was not observed in healthy controls. It is likely that thalidomide has some other pharmacological effects that diminish the exaggerated inflammatory environment that already existed in CGD monocytes [8]. Indeed, diminished inflammation due to thalidomide therapy was observed clinically in our patient with CGC colitis, who recovered following thalidomide therapy concomitant with decreases in serum inflammatory cytokines. Of importance was that the patient experienced no episodes of serious infection during short-term thalidomide therapy. Since granuloma formation is associated with dysregulated TNF-α production in inflammatory lesions [5], thalidomide, which selectively inhibits NF-κB activation induced by TNF-α, seems to be considered as a therapeutic option for patients with CGD colitis who are experiencing serious pathogenic infection.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

We are grateful to the CGD patients and blood donors for their participation in this study. This work was supported by a Grant-in-Aid for Young Scientists (B) and a Grant from the National Center for Child Health and Development.

References

[1] F. Meissner, R.A. Seger, D. Moshous, A. Fischer, J. Reichenbach, A. Zychlinsky, Inflammasome activation in NADPH oxidase defective mononuclear phagocytes from patients with chronic granulomatous disease, Blood 116 (2010) 1570–1573.
[2] C. Dostert, V. Petrilli, R. Van Bruggen, C. Steele, B.T. Mossman, J. Tschopp, Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica, Science 320 (2008) 674–677.
[3] T. Kawai, H. Kusakabe, A. Seki, S. Kobayashi, M. Onodera, Osteomyelitis due to trimethoprim/sulfamethoxazole-resistant *Edwardsiella tarda* infection in a patient with X-linked chronic granulomatous disease, Infection 39 (2011) 171–173.
[4] D.J. Marks, K. Miyagi, F.Z. Rahman, M. Novelli, S.L. Bloom, A.W. Segal, Inflammatory bowel disease in CGD reproduces the clinicopathological features of Crohn's disease, Am. J. Gastroenterol. 104 (2009) 117–124.
[5] G. Uzel, J.S. Orange, N. Poliak, B.E. Marciano, T. Heller, S.M. Holland, Complications of tumor necrosis factor-alpha blockade in chronic granulomatous disease-related colitis, Clin. Infect. Dis. 51 (2010) 1429–1434.
[6] C. Meierhofer, C.J. Wiedermann, New insights into the pharmacological and toxicological effects of thalidomide, Curr. Opin. Drug Discovery Devel. 6 (2003) 92–99.
[7] M. Keller, G. Sollberger, H.D. Beer, Thalidomide inhibits activation of caspase-1, J. Immunol. 183 (2009) 5593–5599.
[8] M. Eski, I. Sahin, M. Sengezer, M. Serdar, A. Ifran, Thalidomide decreases the plasma levels of IL-1 and TNF following burn injury: is it a new drug for modulation of systemic inflammatory response, Burns 34 (2008) 104–108.

[9] H. Sokol, F. Suarez, T. Meatchi, G. Malamut, M.A. Pocidalo, S. Blanche, C. Cellier, O. Hermine, Thalidomide as a treatment for refractory CGD colitis, Am. J. Gastroenterol. 104 (2009) 1069.

[10] M.A. Wallet, S.M. Wallet, G. Guiulfo, J.W. Sleasman, M.M. Goodenow, IFNgamma primes macrophages for inflammatory activation by high molecular weight hyaluronan, Cell. Immunol. 262 (2010) 84–88.

[11] M.G. Netea, C.A. Nold-Petry, M.F. Nold, L.A. Joosten, B. Opitz, J.H. van der Meer, F.L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, C.J. Funk, R.J. Mason, B.J. Kullberg, A. Rubartelli, J.W. van der Meer, C.A. Dinarello, Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages, Blood 113 (2009) 2324–2335.

[12] F.L. van de Veerdonk, S.P. Smeekens, L.A. Joosten, B.J. Kullberg, C.A. Dinarello, J.W. van der Meer, M.G. Netea, Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 3030–3033.

[13] R. Zhou, A.S. Yazdi, P. Menu, J. Tschopp, A role for mitochondria in NLRP3 inflammasome activation, Nature 469 (2011) 221–225.

[14] J. Liobikas, D. Majiene, S. Trumbeckaite, L. Kursvietiene, R. Masteikova, D.M. Kopustinskiene, A. Savickas, J. Bernatoniene, Uncoupling and antioxidant effects of ursolic acid in isolated rat heart mitochondria, J. Nat. Prod. 74 (2011) 1640–1644.

[15] S. Majumdar, B. Lamothe, B.B. Aggarwal, Thalidomide suppresses NF-kappa B activation induced by TNF and H2O2, but not that activated by ceramide, lipopolysaccharides, or phorbol ester, J. Immunol. 168 (2002) 2644–2651.

[16] L. Franchi, R. Munoz-Planillo, G. Nunez, Sensing and reacting to microbes through the inflammasomes, Nat. Immunol. 13 (2012) 325–332.

[17] P. Pelegrin, A. Surprenant, Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor, EMBO J. 25 (2006) 5071–5082.

[18] D. Turner, A.R. Otley, D. Mack, J. Hyams, J. de Bruijne, K. Uusoue, T.D. Walters, M. Zachos, P. Mamula, D.E. Beaton, A.H. Steinhart, A.M. Griffiths, Development, validation, and evaluation of a pediatric ulcerative colitis activity index: a prospective multicenter study, Gastroenterology 133 (2007) 423–432.

[19] K. Yasui, N. Uchida, Y. Akazawa, S. Nakamura, I. Minami, Y. Amano, T. Yamazaki, Thalidomide for treatment of intestinal involvement of juvenile-onset Behcet disease, Inflamm. Bowel. Dis. 14 (2008) 396–400.

[20] S. Malik, S.C. Wong, J. Bishop, K. Hassan, P. McGrogan, S.F. Ahmed, R.K. Russell, Improvement in growth of children with Crohn disease following anti-TNF-alpha therapy can be independent of pubertal progress and glucocorticoid reduction, J. Pediatr. Gastroenterol. Nutr. 52 (2011) 31–37.

[21] T. Olsen, R. Goll, G. Cui, I. Christiansen, J. Florholmen, TNF-alpha gene expression in colorectal mucosa as a predictor of remission after induction therapy with infliximab in ulcerative colitis, Cytokine 46 (2009) 222–227.

[22] E. Hatanaka, B.T. Carvalho, A. Condino-Neto, A. Campa, Hyperresponsiveness of neutrophils from gp 91phox deficient patients to lipopolysaccharide and serum amyloid A, Immunol. Lett. 94 (2004) 43–46.

[23] B.H. Segal, W. Han, J.J. Bushey, M. Joo, Z. Bhatti, J. Feminella, C.G. Dennis, R.R. Vethanayagam, F.E. Yull, M. Capitano, P.K. Wallace, H. Minderman, J.W. Christman, M.B. Sporn, J. Chan, D.C. Vinh, S.M. Holland, L.R. Romani, S.L. Gaffen, M.L. Freeman, T.S. Blackwell, NADPH oxidase limits innate immune responses in the lungs in mice, PLoS One 5 (2010) e9631.

[24] J.A. Keifer, D.C. Guttridge, B.P. Ashburner, A.S. Baldwin Jr., Inhibition of NF-kappa B activity by thalidomide through suppression of IkappaB kinase activity, J. Biol. Chem. 276 (2001) 22382–22387.

Pediatr Blood Cancer 2013;60:885–886

LETTER TO THE EDITOR

Neutropenia and Myeloid Dysplasia in a Patient With Delayed-Onset Adenosine Deaminase Deficiency

To the Editor: Adenosine deaminase (ADA) deficiency typically leads to severe combined immunodeficiency (SCID) presenting in the first few months of life. In the absence of ADA, there is intracellular accumulation of adenosine and deoxyadenosine, which are toxic to lymphocytes and cause severe lymphopenia. Approximately 15% of ADA deficiency cases are diagnosed later than infancy, which are referred to as delayed-onset type. At the age of 2 years, the patient was admitted for acute disseminated encephalomyelitis. At the age of 3 years, laboratory study revealed lymphopenia (580/μl) and neutropenia (600/μl), and he was suspected of primary immunodeficiency because of recurrent infections and autoimmune neutropenia and thyroiditis. The patient showed a decreased level of ADA (1.0 μmol/hours/mg protein; normal, 26.4 ± 10.0 μmol/hours/mg protein) and

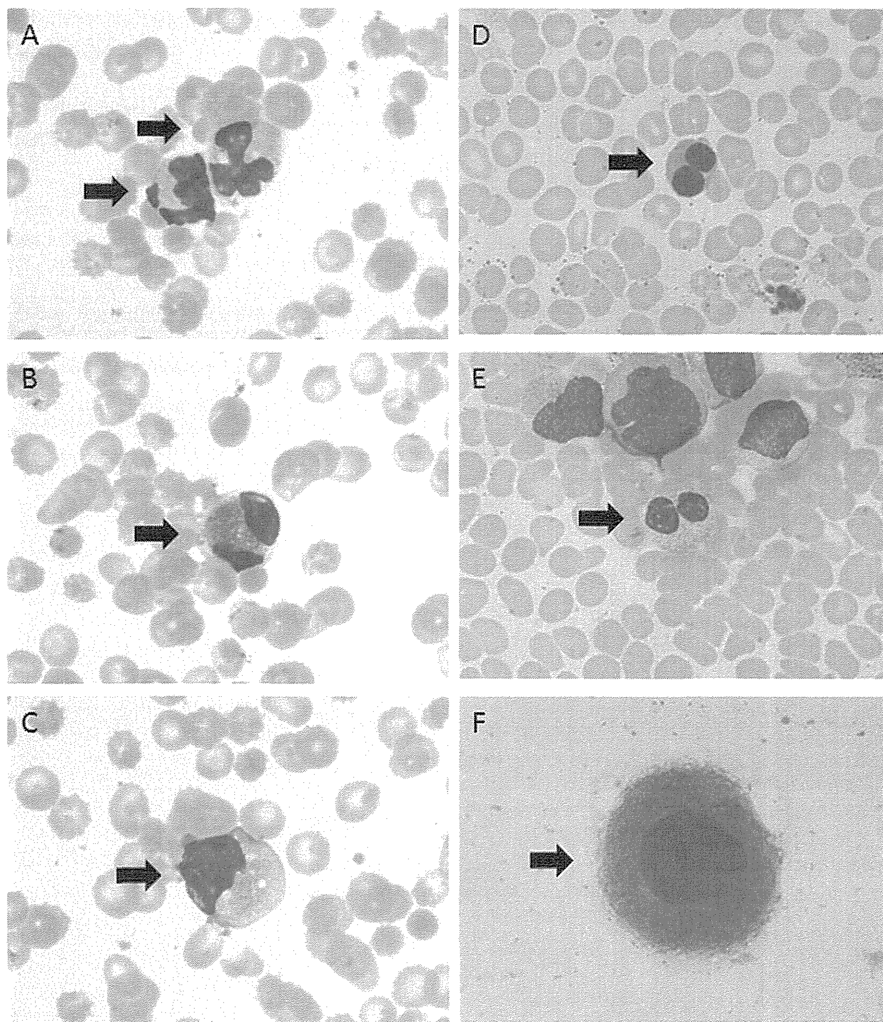

Fig. 1. Blood smear abnormalities in the patient. Blood smear in peripheral blood (**A–C**) and bone marrow (**D–F**). A: Neutrophils with condensed chromatin and hypogranules. B: Eosinophil with abnormal granules. C: Monocyte with condensed chromatin. D: Bi-nucleated erythoblast. E: Neutrophil with pseudo-Pelger–Huet anomaly. F: Small-sized non-lobulated megakaryocyte. Arrows indicate pathological changes. Original magnification ×1,000.

*Correspondence to: Hirokazu Kanegane, MD, PhD, Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama, Toyama 930-0194, Japan. E-mail: kanegane@med.u-toyama.ac.jp

Received 7 August 2012; Accepted 6 September 2012

© 2013 Wiley Periodicals, Inc.
DOI 10.1002/pbc.24353
Published online 17 January 2013 in Wiley Online Library (wileyonlinelibrary.com).

increased levels of adenosine and deoxyadenosine (10.8%; normal, <1%) in whole blood. Therefore, the patient was diagnosed with late-onset ADA deficiency due to compound heterozygous missense mutations in the *ADA* gene (R156C and V177M) [1]. He underwent bone marrow transplantation with reduced-intensity conditioning at the age of 4 years [2]. The patient exhibited normal levels of ADA, adenosine, and deoxyadenosine by day 83.

In part because of the ubiquitous expression of ADA, ADA-deficient SCID is associated with pathologic and clinical findings involving various organs, such as the skeleton and central nervous system. Neither bone deformity nor neurological abnormality were observed in the patient. In addition, myeloid dysplasia and bone marrow hypocellularity in ADA-deficient SCID have been reported [3]. We investigated the morphology and cellularity of peripheral blood smear and bone marrow aspiration from the patient. Peripheral blood smear showed mild morphological changes in neutrophils, eosinophils, and monocytes (Fig. 1A–C). Bone marrow aspiration revealed hypocellularity and mild dysplastic changes of three cell lineages (Fig. 1D–F).

Neutropenia and myeloid dysplasia observed in the patient's peripheral blood might be associated not only with autoantibody to neutrophils, but also with ADA deficiency. More severe abnormalities in the peripheral blood smear than in the bone marrow might be caused by mild ADA deficiency. Myeloid dysplasia should be evaluated even in patients with delayed-onset ADA deficiency.

Keiko Nomura, MD, PhD
Akihiro Hoshino, MD
Toshio Miyawaki, MD, PhD
Department of Pediatrics
Graduate School of Medicine and
Pharmaceutical Sciences
University of Toyama
Toyama, Japan

Asahito Hama, MD, PhD
Seiji Kojima, MD, PhD
Department of Pediatrics
Nagoya University Graduate School of Medicine
Nagoya, Japan

Hirokazu Kanegane, MD, PhD*
Department of Pediatrics
Graduate School of Medicine and
Pharmaceutical Sciences
University of Toyama
Toyama, Japan

REFERENCES

1. Nakaoka H, Kanegane H, Taneichi H, et al. Delayed onset adenosine deaminase deficiency associated with acute disseminated encephalomyelitis. Int J Hematol 2012;95:692–696.
2. Kanegane H, Taneichi H, Nomura K, et al. Successful bone marrow transplantation with reduced intensity conditioning in a patient with delay-onset adenosine deaminase deficiency. Pediatr Transplant 2012; Jul 17 [Epub ahead of print].
3. Sokolic R, Maric I, Kesserwan C, et al. Myeloid dysplasia and bone marrow hypocellularity in adenosine deaminase-deficient severe combined immune deficiency. Blood 2011;118:2688–2694.

Pediatr Transplantation 2014: 18: E25–E30

© 2013 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd

Pediatric Transplantation
DOI: 10.1111/petr.12184

Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome

Horino S, Sasahara Y, Sato M, Niizuma H, Kumaki S, Abukawa D, Sato A, Imaizumi M, Kanegane H, Kamachi Y, Sasaki S, Terui K, Ito E, Kobayashi I, Ariga T, Tsuchiya S, Kure S. Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome.

Abstract: IPEX syndrome is a rare and fatal disorder caused by absence of regulatory T cells (Tregs) due to congenital mutations in the Forkhead box protein 3 gene. Here, we report a patient with IPEX syndrome treated with RIC followed by allogeneic BMT from an HLA-matched sibling donor. We could achieve engraftment and regimen-related toxicity was well tolerated. Although the patient was in mixed chimera and the ratio of donor cells in whole peripheral blood remained relatively low, selective and sustained expansion of Tregs determined as CD4+CD25+Foxp3+ cells was observed. Improvement in clinical symptoms was correlated with expansion of donor-derived Tregs and disappearance of anti-villin autoantibody, which was involved in the pathogenesis of gastrointestinal symptoms in IPEX syndrome. This clinical observation suggests that donor-derived Tregs have selective growth advantage in patients with IPEX syndrome even in mixed chimera after allogeneic BMT and contribute to the control of clinical symptoms caused by the defect of Tregs.

Satoshi Horino[1,2], Yoji Sasahara[1], Miki Sato[1], Hidetaka Niizuma[1], Satoru Kumaki[1], Daiki Abukawa[3], Atsushi Sato[2], Masue Imaizumi[2], Hirokazu Kanegane[4], Yoshiro Kamachi[5], Shinya Sasaki[6], Kiminori Terui[6], Etsuro Ito[6], Ichiro Kobayashi[7], Tadashi Ariga[7], Shigeru Tsuchiya[1] and Shigeo Kure[1]

[1]Department of Pediatrics, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan, [2]Department of Hematology and Oncology, Miyagi Children's Hospital, Sendai, Miyagi, Japan, [3]Department of General Pediatrics, Miyagi Children's Hospital, Sendai, Miyagi, Japan, [4]Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Science, University of Toyama, Toyama, Toyama, Japan, [5]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan, [6]Department of Pediatrics, Hirosaki University School of Medicine, Hirosaki, Aomori, Japan, [7]Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan

Key words: allogeneic hematopoietic stem cell transplantation – enteropathy – Forkhead box protein 3 – immune dysregulation – polyendocrinopathy – reduced intensity conditioning – regulatory T cells – X-linked syndrome

Yoji Sasahara, Department of Pediatrics, Tohoku University Graduate School of Medicine, 1-1 Seiryou-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan
Tel: +81 22 717 7287
Fax: +81 22 717 7290
E-mail: ysasahara@med.tohoku.ac.jp

Accepted for publication 24 September 2013

Abbreviations: ALL, acute lymphoblastic leukemia; APC, allophycocyanin; ATG, antithymocyte globulin; BMT, bone marrow transplantation; CyA, cyclosporine A; DAB, 3, 3′-diaminobenzidine; DLI, donor leukocyte infusion; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; IVIG, intravenous immunoglobulin; MLL, mixed lineage leukemia; PBMCs, peripheral blood mononuclear cells; PBSCT, peripheral blood stem cell transplantation; PE, phycoerythrin; PSL, prednisolone; RIC, reduced intensity conditioning; TBI, total body irradiation.

IPEX syndrome is primary immunodeficiency caused by the defects of regulatory T cells (Tregs). IPEX syndrome is often lethal in the first few months of life due to severe diarrhea associated with refractory enteropathy, infections, diabetes mellitus, dermatitis, and other autoimmune complications. This disorder is caused by mutations of Forkhead box protein 3 (*FOXP3*) gene located on chromosome Xp11.23. *FOXP3* encodes Forkhead box protein 3, which is essential for the development and maintenance of CD4+CD25+Foxp3+ Tregs (1, 2).

Established treatments for patients with IPEX syndrome include immunosuppressive therapy and allogeneic HSCT (3–6). Allogeneic HSCT serves as a curative therapy for patients with IPEX syndrome, and RIC regimens have been reported and resulted in better outcome than myeloablative conditioning regimen (7–11). In general, allogeneic HSCT with RIC regimen may increase the risk of rejection and mixed chimera. Some RIC regimens included the antibody against T lymphocytes such as alemtuzumab or ATG. However, these agents may increase the risk of viral reactivation after HSCT. To the best of our knowledge, only two cases of IPEX patients treated with allogeneic HSCT following RIC consisted of low-dose TBI instead of alemtuzumab or ATG have been reported (12).

Here, we report a patient with IPEX syndrome treated with RIC regimen consisted of fludarabine, cyclophosphamide, and low-dose TBI followed by allogeneic HSCT from an HLA-identical sibling donor. Although the patient was in mixed chimera, he was free from symptoms caused by the absence of Tregs. We could observe selective and sustained growth advantage of donor-derived Tregs and disappearance of anti-villin autoantibody in his serum, which was correlated with the improvement in refractory enteropathy.

Patient and methods

Patient

A Japanese male suffered from severe diarrhea at two months of age. He was diagnosed as IPEX syndrome by identifying a missense mutation of T1117G substitution in exon 10 of the *FOXP3* gene (13). We quantified CD4+CD25+Foxp3+ cells by flow cytometry, and positive cells were not identified at all in PBMCs. Autoantibodies examined were negative except anti-villin antibody in patient's serum. He was treated with immunosuppressive therapy of intravenous CyA and oral PSL. After complete remission was achieved, he was free from the symptom for six yr with oral low-dose CyA and PSL (14).

At the age of six, the patient suffered from severe diarrhea again and was referred to our hospital. Although he was treated with increased doses of CyA and PSL in addition to other immunosuppressive agents, these treatments were not effective enough to control his diarrhea completely. We next tried IVIG therapy, which resulted in the improvement in diarrhea, and we could taper immunosuppressive agents.

To control the disease without continuous immunosuppressive therapy, we considered to perform allogeneic BMT from an HLA-matched healthy sibling donor. The donor did not have the mutation in *FOPX3* gene. We used a RIC regimen consisted of 4 Gy (2 × 2 Gy) TBI (day 7), fludarabine at a dose of 30 mg/m^2 for five days (days 6 to day 2) and cyclophosphamide at a dose of 60 mg/kg for two days (days 3 and 2). Total nucleated bone marrow cells of 4.32 × 10^8/kg were transplanted. We selected CyA and short-term methotrexate as GVHD prophylaxis, and IVIG was continued weekly until autoimmune colitis was resolved.

Chimerism assay

Chimerism assay was performed by polymerase-chain-reaction-based assays analyzing polymorphic short tandem repeat markers (15). The chimerism was examined in each fraction of T cells, total lymphocytes, and granulocytes in bone marrow or peripheral blood. We evaluated the chimerism in bone marrow before day 100 and in peripheral blood after day 100, because we had similar results in both samples before day 100 in the patient and avoided frequent bone marrow aspiration after day 100.

Flow cytometry

PBMCs were stained with monoclonal antibodies of APC-conjugated human CD4, PE-conjugated human CD25, and FITC-conjugated human Foxp3 antibodies (BD Biosciences, San Jose, CA, USA) and analyzed by a FACSCanto II flow cytometer (BD Biosciences), as described previously (16).

Immunoblot analysis of anti-villin antibody

Anti-villin autoantibody in patient's serum was analyzed as described previously (17). Briefly, 500 ng of GST-villin recombinant protein (121 kD) was transferred to the membrane and incubated with diluted serum at 1:160. Anti-villin antibody bound to GST-villin was detected by horseradish peroxidase-conjugated antibody and DAB system.

Case report

Clinical improvement after RIC and allogeneic HSCT

The patient achieved an engraftment on day 11, and the last transfusion of platelets was on day 7 and that of red blood cells was on day 1. He was complicated with transient acute GVHD of the skin (grade I) on day 35 but this resolved without additional immunosuppressive therapy. He had no episodes of significant infection and other severe regimen-related toxicity during the course of RIC and allogeneic HSCT.

Severe and bloody diarrhea settled down on day 14 after engraftment. The patient was

consistently free from symptoms of enteropathy and any other autoimmune diseases. Laboratory findings showed improvement in hypoalbuminemia and anemia caused by severe enteropathy on day 21. Colonoscopy examination on day 60 revealed disappearance of mucosal inflammation, multiple ulcerations and hemorrhage that were observed before the HSCT.

After the discharge on day 120, we had followed the patient every two wk. He had no episodes of autoimmune disorders and infection, and we could taper and stop immunosuppressive agents at six months. Unfortunately, he suffered from MLL gene-rearranged ALL at 24 months after transplantation. The origin of precursor B lymphoblasts was recipient cells. We treated him with chemotherapy and allogeneic PBSCT from the same donor. We used myeloablative conditioning regimen consisted of busulfan at a dose of 4 mg/kg for four days and melphalan at a dose of 90 mg/m^2 for two days for the second transplant from the same sibling donor to cure this secondary ALL. He has been in complete remission for more than two yr. Chimerism completely changed to donor-type and the number of Tregs increased to normal after the second transplant.

Chimerism and immunological evaluation after first allogeneic HSCT

Because the ratio of donor T cells, total lymphocytes, and granulocytes in bone marrow was 74%, 48%, and 48%, respectively, on day 22 after HSCT, we reduced the dose of CyA immediately. The ratio of donor cells, however, was further declined to 5% on day 50, and the donor bone marrow was assumed to be rejected (Fig. 1). At that point, flow cytometric analysis of peripheral blood showed that 17.8% of PBMCs were CD4+CD25+Foxp3+ Tregs (Fig. 2a,b). This discordant result on day 50 was explained by selective expansion of donor-derived Tregs. After discontinuation of CyA on day 50, the ratio of donor T cells, total lymphocytes, and granulocytes was transiently increased up to 40% and then gradually decreased (Fig. 1). At 24 months after HSCT, donor cells were around 20% and CD4+CD25+Foxp3+ Tregs were at the range of 1.2–3.0% of PBMCs, which were comparable to healthy controls (Fig. 2a). We did not perform DLI because the ratio of donor cells was <50% and supposed that the patient was in high risk of bone marrow aplasia after repeated DLI.

The anti-villin autoantibody was detected by immunoblot analysis when the disease was active before HSCT. The antibody was under detectable levels both in clinical remission by immunosuppressive therapy before HSCT and after engraftment was achieved following HSCT even when immunosuppressive agents were not administrated (Table 1).

Discussion

The defect of Tregs in patients with IPEX syndrome causes symptoms related to

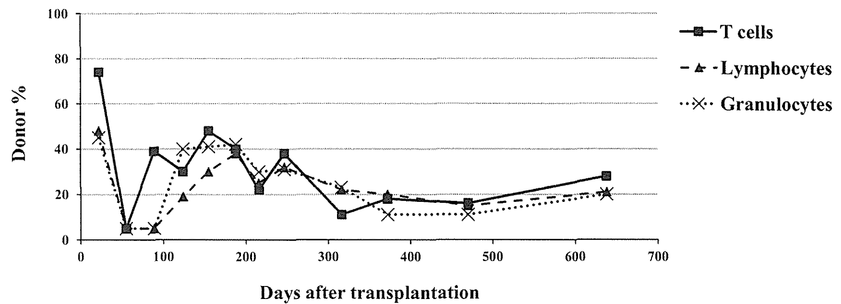

Fig. 1. Frequency of donor cells after allogeneic HSCT. Percentages of donor cells in each fraction of T cells, total lymphocytes, and granulocytes after HSCT are shown.

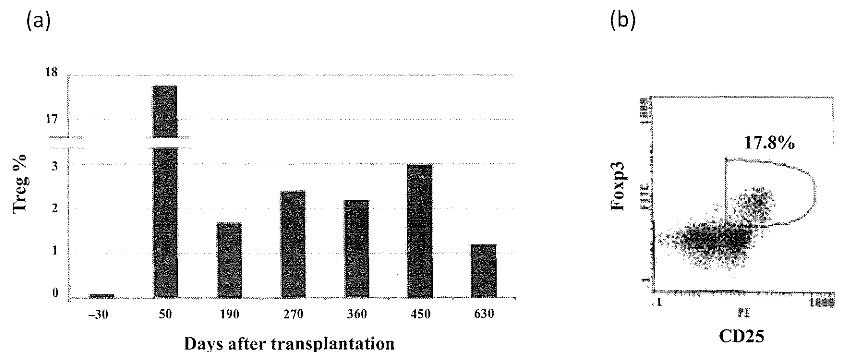

Fig. 2. Sustained expansion of donor Tregs after allogeneic HSCT. (a) Percentages of Tregs are evaluated as CD4+CD25+Foxp3+ cells in PBMCs. (b) Flow cytometric demonstration of CD25+Foxp3+ cells in CD4 gated PBMCs on day 50 after HSCT.

Table 1. Anti-villin autoantibody is correlated with clinical condition of enteropathy

Clinical condition of enteropathy	Anti-villin autoantibody
Disease onset	+
Remission before HSCT	−
Relapse before HSCT	+
After HSCT	−

GST-villin protein (121 kD) was transferred to the membrane, and immunoblot was performed with 1:160 diluted patient's serum at different disease condition of enteropathy as indicated. + indicates the presence of anti-villin antibody that recognizes GST-villin protein.

autoimmunity. However, clinical benefit of immunosuppressive therapy is often limited by its adverse effects and increased susceptibility to infection. At present, allogeneic HSCT is recognized as the curative therapy for patients with IPEX syndrome. We summarized all reported cases treated with HSCT in Table 2. Myeloablative regimen resulted in high fatality due to regimen-related toxicity or lethal infection (2, 5, 6). On the other hand, Rao et al. (7) first reported four patients who were successfully treated with non-myeloablative conditioning regimen consisted of fludarabine, melphalan, and alemtuzumab, and achieved high rate of donor chimerism above 84.6%. Non-myeloablative regimens with anti-T-lymphocyte antibody such as alemtuzumab or ATG have been used, and all patients are alive (7–11). However, it is known that alemtuzumab and ATG induce profound depletion of T cells and increase the risk of viral reactivation and fungal infection after HSCT. Therefore, we used low-dose TBI instead of anti-T-lymphocyte antibodies. The combination of low-dose TBI, fludarabine, and cyclophosphamide was well tolerated, and the patient was free from infections and severe regimen-related toxicities. Burroughs et al. (12) also reported that RIC regimen including low-dose TBI for IPEX syndrome resulted in stable engraftment of Tregs and better clinical outcome, proposing that this regimen was preferable for patients with IPEX syndrome.

The patient developed MLL-related secondary ALL in recipient cells. Although the dose of TBI was less than used in myeloablative conditioning, radiation and alkylating agents might cause DNA damage and increased the risk of secondary leukemia in recipient cells. Alternatively, the use of anti-T-lymphocyte antibody instead of low-dose TBI and/or dose reduction in alkylating agents should be carefully considered in IPEX syndrome.

Selected and sustained expansion of Tregs resulted in clinical improvement even though the patient was in mixed chimera after HSCT. Seidel et al. (11) reported a patient with IPEX

Table 2. Summary of IPEX patients treated with allogeneic HSCT reported in the literature

Case	Age	Donor	Conditioning regimen	Complications after HSCT	Outcome	% Donor after HSCT	Reference
1	13 yr	HLA-matched sibling	TBI 12 Gy + CY + ATG	Adenovirus infection, pneumonia	Dead	50%	2
2	9 yr	HLA-matched unrelated	TBI 12 Gy + CY + ATG	Cytomegalovirus infection, hemorrhagic cystitis, lymphoproliferative disorder	Dead	70%	2
3	4 months	HLA-matched sibling	BU + CY + ALG	Hemophagocytic syndrome	Dead	30% in T cell	5
4	1 yr	HLA-matched sibling	BU + CY + Flu + ATG		Alive	70% in T cell	6
5	7 yr	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Cytomegalovirus infection	Alive	100%	7
6	1 yr	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Acute respiratory distress syndrome	Alive	100%	7
7	4 yr	HLA-matched sibling	Flu + L-PAM + alemtuzumab	Histoplasma infection	Alive	89%	7
8	5 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab		Alive	84.6%	7
9	7 yr	HLA 5/6-matched cord blood	Flu + BU + ATG	Lymphoproliferative disorder	Alive	81 ~ 98%	8
10	7 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Sepsis of *Enterobacter cloacae*	Alive	100%	9
11	5 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab + anti-CD 45 monoclonal antibody		Alive	100%	10
12	11 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab		Alive	<10%	11
13	9 months	HLA-matched unrelated	TBI 4 Gy + Flu	Bacteremia	Alive	100%	12
14	16 yr	HLA-matched related	TBI 4 Gy + Flu	Bacteremia	Alive	20 ~ 60% in T cell	12

Case series transplanted with RIC regimens were highlighted.
CY, cyclophosphamide; ALG, antilymphocyte globulin; BU, busulfan; Flu, fludarabine; L-PAM, melphalan.

syndrome who showed selective engraftment of Tregs for six yr after non-myeloablative transplantation. It has been reported that partial BMT or injection of T-enriched splenocytes resulted in the rescue of autoimmunity in Scurfy mice, a mouse model for IPEX syndrome in which *FOXP3* gene is naturally mutated. Sustained engraftment of relatively high frequency of CD4+CD25+Foxp3+ Tregs was observed even though the frequency of donor cells in whole peripheral blood ranged from 1.7% to 50% (18). These observations illustrate that the paradigm in the generation of Tregs is reinforced by the requirement and growth advantage regardless of chimerism of other hematopoietic cells in IPEX syndrome. However, we should still consider the possibility that mixed chimerism may result in subsequent development of autoimmune diseases observed in other primary immunodeficiency, as previously reported in some patients with Wiskott–Aldrich syndrome (19).

Intractable diarrhea is a major symptom in patients with IPEX syndrome. Villin, an actin-binding protein, is expressed as the 95 kD antigen in the small intestine, which is frequently targeted by autoantibodies in patients with IPEX syndrome (17). Anti-villin antibody was clearly correlated with the severity of clinical symptoms in our patient. Therefore, monitoring of anti-villin antibody might serve as a useful examination for evaluating gastrointestinal complications in patients with IPEX syndrome.

We reported here a unique phenomenon of selective growth advantage of Tregs in a patient with IPEX syndrome who was in mixed chimera after RIC and allogeneic HSCT. Sustained expansion of donor-derived Tregs resulted in the significant improvement in enteropathy. To determine optimal RIC regimen to achieve complete chimera and avoid secondary malignancy in residual recipient cells, further analysis in more patients and long-term follow-up study after HSCT are required to conclude this issue.

Authors' contributions

Horino S and Sasahara Y designed the study, interpreted the data, wrote the paper, and treated the patient. Sato M, Kanegane H, Kamachi Y, and Kobayashi I performed experiments. All other authors treated the patient and collected clinical data.

Acknowledgments

We thank all of our colleagues in the Department of Pediatrics, Tohoku University, who contributed to the patient's care. This work was supported by the grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23591528 to YS), a grant for research on intractable diseases from the Ministry of Health, Labour and Welfare of Japan (to YS), grants from Japan Leukemia Research Fund and SENSHIN Medical Research Foundation (to YS).

Conflict of interest disclosure

The authors declare no conflict of interest.

References

1. Powell BR, Bruist NR, Stenzel P. An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy. J Pediatr 1982: 100: 731–737.
2. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome. J Med Genet 2002: 39: 537–545.
3. Taddio A, Faleschini E, Valencic E, et al. Medium-term survival without hematopoietic stem cell transplantation in a case of IPEX: Insights into nutritional and immunosuppressive therapy. Eur J Pediatr 2007: 166: 1195–1197.
4. Gambineri E, Perroni L, Passerini L, et al. Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: Inconsistent correlation between forkhead box protein 3 expression and disease severity. J Allergy Clin Immunol 2008: 122: 1105–1112.
5. Baud O, Goulet O, Canioni D, et al. Treatment of the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) by allogeneic bone marrow transplantation. N Engl J Med 2001: 344: 1758–1762.
6. Mazzolari E, Forino C, Fontana M, et al. A new case of IPEX receiving bone marrow transplantation. Bone Marrow Transplant 2005: 35: 1033–1034.
7. Rao A, Kamani N, Filopovich A, et al. Successful bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. Blood 2007: 109: 383–385.
8. Lucas KG, Ungar D, Comito M, et al. Submyeloablative cord blood transplantation corrects clinical defects seen in IPEX syndrome. Bone Marrow Transplant 2007: 39: 55–56.
9. Dorsey MJ, Petrovic A, Morrow MR, et al. FOXP3 expression following bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. Immunol Res 2009: 44: 179–184.
10. Zhan H, Sinclair J, Adams S, et al. Immune reconstitution and recovery of FOXP3 (forkhead box protein 3)-expressing T cells after transplantation for IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome. Pediatrics 2008: 121: 998–1002.
11. Seidel MG, Fritsch G, Lion T, et al. Selective engraftment of donor $CD4^{+}CD25^{high}$ FOXP3-positive T cells in IPEX syndrome after nonmyeloablative hematopoietic stem cell transplantation. Blood 2009: 113: 5689–5691.
12. Burroughs LM, Torgerson TR, Storb R, et al. Stable hematopoietic cell engraftment after low-intensity nonmyeloablative conditioning in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. J Allergy Clin Immunol 2010: 126: 1000–1005.
13. Tanaka H, Suzuki K, Nakahata T, et al. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Acta Paediatr 2004: 93: 142–143.
14. Tanaka H, Tsugawa K, Kudo M, et al. Low-dose cyclosporine A in a patient with X-linked immune dysregulation, polyendocrinopathy and enteropathy. Eur J Pediatr 2005: 164: 779–780.

15. Thiede C, Florek M, Bornhäuser M, et al. Rapid quantification of mixed chimerism using multiplex amplification of short tandem repeat markers and fluorescence detection. Bone Marrow Transplant 1999: 23: 1055–1060.
16. Otsubo K, Kanegane H, Kamachi Y, et al. Identification of FOXP3-negative regulatory T-like ($CD4^+CD25^+CD127^{low}$) cells in patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. Clin Immunol 2011: 141: 111–120.
17. Kobayashi I, Kubota M, Yamada M, et al. Autoantibodies to villin occur frequently in IPEX, a severe immune dysregulation, syndrome caused by mutation of FOXP3. Clin Immunol 2011: 141: 83–89.
18. Smyk-Pearson SK, Bakke AC, Held PK, et al. Rescue of the autoimmune scurfy mouse by partial bone marrow transplantation or injection with T-enriched splenocytes. Clin Exp Immunol 2003: 133: 193–199.
19. Ozsahin H, Cavazzana-Calvo M, Notarangelo LD, et al. Long-term outcome following hematopoietic stem-cell transplantation in Wiskott-Aldrich syndrome: Collaborative study of the European Society for Immunodeficiencies and European Group for Blood and Marrow Transplantation. Blood 2008: 111: 439–445.

J Clin Immunol
DOI 10.1007/s10875-013-9964-4

ASTUTE CLINICIAN REPORT

Allogeneic Bone Marrow Transplantation Appears to Ameliorate IgA Nephropathy in a Patient with X-linked Thrombocytopenia

Akihiro Hoshino · Masaki Shimizu · Hiroyoshi Matsukura · Hisano Sakaki-Nakatsubo · Keiko Nomura · Toshio Miyawaki · Hirokazu Kanegane

Received: 24 February 2013 / Accepted: 29 October 2013
© Springer Science+Business Media New York 2013

Abstract Wiskott-Aldrich syndrome (WAS) is caused by a mutation in the *WAS* gene, and it is clinically characterized by the triad of thrombocytopenia, eczema and immunodeficiency. X-linked thrombocytopenia (XLT), which is a clinically mild form of WAS, is also caused by a *WAS* gene mutation. Patients with WAS/XLT sometimes also have autoimmune diseases such as IgA nephropathy. Progression of IgA nephropathy may lead to chronic renal failure with a poor prognosis. Here, we describe an XLT patient who also had IgA nephropathy. The patient underwent bone marrow transplantation (BMT) because of an associated-lymphoproliferative disorder, and clinical and histological improvement in his IgA nephropathy was observed after BMT. The amount of galactose-deficient IgA in the patient's serum markedly decreased after BMT. Therefore, immunological reconstitution might improve autoimmune diseases in patients with WAS/XLT.

Keywords Aberrant IgA · bone marrow transplantation · IgA nephropathy · Wiskott-Aldrich syndrome · X-linked thrombocytopenia

A. Hoshino · H. Sakaki-Nakatsubo · K. Nomura · T. Miyawaki · H. Kanegane (✉)
Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
e-mail: kanegane@med.u-toyama.ac.jp

M. Shimizu
Department of Pediatrics, School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan

H. Matsukura
Department of Pediatrics, Saiseikai Toyama Hospital, Toyama, Japan

Published online: 12 November 2013

Introduction

Wiskott-Aldrich syndrome (WAS) is clinically characterized by thrombocytopenia with small platelets, eczema, and humoral and cellular immunodeficiency, and it is caused by a mutation in the *WAS* gene and deficient expression of WAS protein (WASP) [1]. The molecular defect in *WAS* also results in X-linked thrombocytopenia (XLT), which is a clinically mild form of WAS [2]. Patients with WAS exhibit an increased incidence of autoimmune diseases, in which vasculitis, autoimmune hemolytic anemia and kidney disease are the most common manifestations [3]. Recently, a study involving a large cohort of patients with XLT revealed that autoimmune diseases are also frequently observed in XLT [4].

Kidney disease, the majority of which is IgA nephropathy, is found in 4–19 % and 5 % of patients with WAS [3, 5] and XLT [4], respectively. IgA nephropathy is a glomerulonephritis that is immunohistochemically characterized by mesangial proliferation with diffuse IgA deposition [6]. Multiple pathogeneses of IgA nephropathy, including impaired response to mucosal antigens, delayed clearance of immune complexes from the circulation and abnormal interaction with mesangial IgA receptors, have been reported [7]. In the above hypotheses, abnormal glycosylation of the IgA1 molecule plays a critical role [6, 7]. The level of poorly galactosylated IgA1 in serum was found to be increased in patients with IgA nephropathy [8]. Aberrant IgA had an increased tendency to self-aggregate and form immune complexes with IgG antibodies, and it is therefore more likely to be deposited in the mesangium [6]. In *WASP*-deficient mice, the increased production of aberrant IgA was strongly related to the development of IgA nephropathy [9].

The reason that aberrant IgA is produced in WAS/XLT remains unclear, but immunological dysfunction may play an important role [10–12]. Therefore, there is a possibility

that immune reconstitution improves IgA nephropathy. Our XLT patient demonstrated clinical and histochemical improvement in IgA nephropathy following bone marrow transplantation (BMT).

Material and Methods

Patient

The patient is a 19-year-old male, and he developed a subcutaneous hematoma at the age of 3 years, and was found to have thrombocytopenia. The family history showed that his maternal uncle had thrombocytopenia and IgA nephropathy, which resulted in end-stage renal failure at the age of 30 years. At the age of 6 years, the patient was diagnosed as having an XLT (G1487A).

Subsequently, he remained well, and his platelet count fluctuated between 50 and $100\times10^3/\mu L$. At the age of 8 years, the patient showed gross hematuria and proteinuria after an upper respiratory infection and was suspected to have glomerulonephritis (Table I). A percutaneous renal biopsy was performed, and he was diagnosed with IgA nephropathy [13]. He was treated with an angiotensin-converting enzyme inhibitor and low-dose prednisolone (PSL) for one year. He improved only partially, and the hematuria and proteinuria persisted. At the age of 14 years, he contracted an Epstein-Barr virus-negative atypical lymphoproliferative disorder and achieved remission after receiving 1 mg/kg per day of PSL.

Following the remission of lymphoproliferative disease, the patient underwent a BMT from an unrelated human leukocyte antigen (HLA)-matched donor. The pre-BMT conditioning regimen included busulfan (12.8 mg/kg), cyclophosphamide (200 mg/kg), rituximab (375 mg/m^2) and 3 Gy of total body irradiation. Tacrolimus (day -1 to 30, 0.01 mg/kg/day administered with continuous infusion, target serum levels of 10–15 ng/mL; from day 31 converted to oral at 0.03 mg/kg/day, target trough serum levels of 5 ng/mL), methotrexate (day 1, 15 mg/m^2; day 3, 10 mg/m^2) were used for graft-versus-host disease (GVHD) prophylaxis. Tacrolimus was tapered from 12 months after BMT and discontinued 20 months after BMT. PSL (0.2–0.5 mg/kg/day) was used for engraftment syndrome and GVHD for 8 months. The patient had complete chimerism soon after BMT. A second renal biopsy was performed 24 months after BMT (Table I).

Measurement of Lectin-Binding Serum IgA Levels

To examine the terminal galactosylation of IgA molecules, we used lectin-binding assays with Helix aspersa (HAA), which

Table I Laboratory findings of patient 1 before and after BMT

Test	At the first biopsy	1 year after low-dose PSL	Just before HSCT	At the second biopsy	Value
WBC	12,110	8,280	8,020	7,220	/μL
RBC	431	436	487	472	$\times10^4/\mu L$
Hemoglobin	13.9	12.4	14.5	14.5	g/dL
Hematocrit	39.1	35.4	41.6	42.4	%
Platelets	82	49	158	232	$\times10^3/\mu L$
BUN	9	10	14	7	mg/dL
Creatinine	0.5	0.5	0.8	0.6	mg/dL
IgG	613	916	899	1,060	mg/dL
IgA	264	315	261	131	mg/dL
IgM	23	18	116	267	mg/dL
CH50	34	NE	NE	NE	U/mL
C3	105	NE	NE	NE	mg/dL
C4	35	NE	NE	NE	mg/dL
Urinalysis					
Protein	2+	2+	2+	neg	
Occult blood	3+	+	+	neg	
RBC	100-150/HPF	10-19/HPF	10-19/HPF	neg	
WBC	5-10/HPF	5-10/HPF	neg	neg	
Hyaline cast	+	neg	neg	neg	
Granular cast	+	neg	1-4/WF	1-4/WF	

WBC white blood cells, *RBC* red blood cells, *BUN* blood urea nitrogen, *Ig* immunoglobulin, *CH50* complement activity, *NE* not examined, *C* complement, *HPF* high power field, *WF* whole field

recognizes terminal N-acetylgalactosamine (GalNAc) residues [14]. The ratio of the absorbance at 450 nm between IgA bound to HAA and total IgA (HAA/IgA) was calculated. The amount of galactose-deficient IgA in serum was estimated using the following formula: serum IgA concentration (C_{IgA})×HAA/IgA ratio.

Results

The first renal biopsy revealed that all of 28 glomeruli showed diffuse mesangial hypercellularity, and 4 glomeruli were completely scarred-in (Fig. 1a). Immunofluorescence studies showed IgA and IgG deposits were present, mainly in the mesangium (Fig. 1b and c). Electron microscopy showed a cluster of electron-dense deposits in the mesangium (Fig. 1g). The second renal biopsy showed that all 14 glomeruli were normal without mesangial proliferation, although 2 glomeruli showed mesangial sclerosis (Fig. 1d). IgA and IgG deposits significantly improved in immunofluorescent studies (Fig. 1e and f). Electron microscopy confirmed the disappearance of electron-dense deposits (Fig. 1h).

The IgA-dominant immune deposits in the mesangium and glomerular injury in the patient significantly improved after BMT. The amount of galactose-deficient IgA in his serum also markedly decreased after BMT (before BMT; 126.4 mg/dL, after BMT; 36.6 mg/dL) [14].

Discussion

This may be the first report to provide a detailed description of IgA nephropathy associated with WAS/XLT after BMT. The clinical course of the patient indicates that BMT can improve the IgA nephropathy associated with WAS/XLT. Although the detail was not described, a patient with IgA nephropathy and chronic myelogenous leukemia has been reported who had a remission not only of the leukemia but also of the IgA nephropathy after BMT [15]. In murine models of IgA nephropathy, IgA deposition in the glomerular lesion decreased after BMT from quiescent murine [16]. Conversely, BMT from the onset murine to quiescent murine resulted in IgA deposition in the glomerular lesion [17]. This study suggests that hematopoietic stem cells or their differentiated cells are related to IgA nephropathy. Recently, abnormalities of lymphocytes, which may be related to the production of aberrant IgA, have been reported [18–24]. B cells show abnormal activity of β-1,3-galactosyltransferase or α-2,6-sialyltransferase, which is the enzyme associated with glycosylation of the IgA1 molecule [18–20] and abnormal regulation of Toll-like receptors [21,

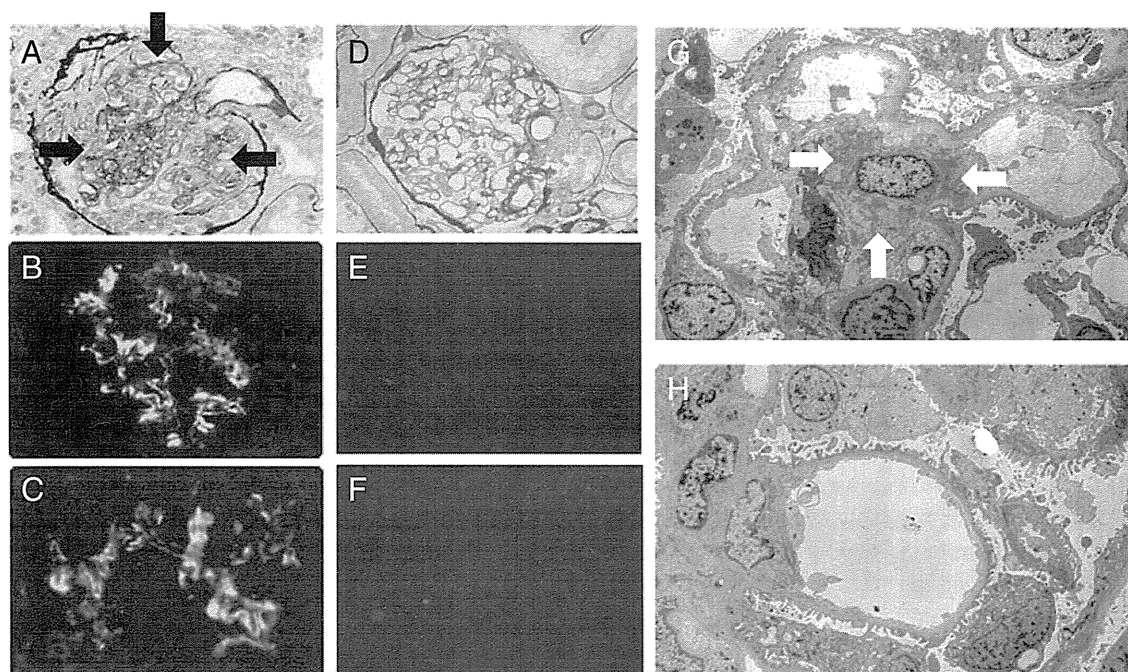

Fig. 1 Histological findings of renal biopsy samples from patient 1 before and after BMT. Histology of renal biopsy before (**a**, **b**, **c** and **g**) and after BMT (**d**, **e**, **f** and **h**). Periodic acid-methenamine-silver staining (**a** and **d**). Immunofluorescence studies of IgA (**b** and **e**) and IgG (**c** and **f**). Electron microscopy (**g** and **h**). Diffuse mesangial proliferative glomerulonephritis was observed (*arrows*) (**a**), and IgA and IgG deposits were observed in the mesangium (**b** and **c**) before BMT. Electron microscopy showed a cluster of electron-dense deposits in the mesangium (*arrows*) (**g**). Little proliferation of mesangium and cells was observed (**d**), and IgA, IgG and electron-dense deposits disappeared (**e**, **f** and **h**) after BMT

22]. T cells show abnormal secretion of IL-17 and T helper (Th) 2 cytokines in IgA nephropathy [19, 23].

The reason for the production of aberrant IgA and the development of IgA nephropathy in WAS/XLT remains unclear, but abnormalities of lymphocytes may play an important role. Consistent with previous reports, decreased levels of aberrant IgA and improvement of IgA nephropathy after BMT were observed in this patient. It has been reported that aberrant IgA and B cells which produce aberrant IgA increase age-dependently [9, 14], but there are no reports of mechanisms responsible for the aberrant glycosylation of IgA in WAS patients. However, impaired function of natural regulatory T cells and imbalance of Th1/Th2 cytokines, which are shown in IgA nephropathy, are also shown in WAS patients [10–12].

Histological improvement of IgA nephropathy might be caused by the clearance of mesangial IgA and the prevention of new IgA deposits. In renal transplantation, the deposits of mesangial IgA rapidly disappear after transplantation from donors with IgA nephropathy into recipients with non-IgA related disease [24, 25]. These observations seem to reflect a similar histological improvement. However, the precise mechanism remains unclear.

We suggest that hematopoietic stem cell transplantation (HSCT) for XLT patients can improve the long-term renal prognosis. HSCT at an early age is the treatment of choice for WAS patients [1]. However, therapeutic options for patients with XLT are controversial because of the excellent survival [4]. However, patients with XLT show a high rate of severe disease-related complications, and the prevalence of IgA nephropathy is 5 %; this condition occurs more frequently in Japanese patients, in whom the rate is 18 % [4]. Autoimmune diseases are significantly related to mixed/split chimerism for patients with WAS/XLT, suggesting that residual host lymphocytes or altered cytoskeleton can cause autoimmune diseases [26]. Therefore, adequate myeloablation and immunosuppression may be required.

Animal studies and the success in our patient support that HSCT may improve IgA nephropathy. Although in our patient, we cannot fully exclude the benefit of the myeloablative chemotherapy and post-transplant immunosuppressive GVHD-prophylaxis medications. Further studies are needed to define the role of the affected immune system in XLT and WAS in promoting IgA nephropathy, as well as, the role of immune reconstitution in improvement of the disease. Regardless, based on the supporting data from animal studies and our patient's beneficial result, HSCT should be considered for improving the clinical outcome in patients with XLT and IgA nephropathy.

Acknowledgments This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (to H. K. and T. M.), and grants from the Ministry of Health, Labour and Welfare of Japan (to T. M.). We thank Ms. Chikako Sakai and Mr. Hitoshi Moriuchi for their excellent technical assistance.

References

1. Ochs HD, Thrasher AJ. The Wiskott-Aldrich syndrome. J Allergy Clin Immunol. 2006;117:725–38.
2. Villa A, Notarangelo L, Macchi P, Mantuano E, Cavagni G, Brugnoni D, et al. X-linked thrombocytopenia and Wiskott-Aldrich syndrome are allelic diseases with mutations in the WASP gene. Nat Genet. 1995;9:414–7.
3. Imai K, Morio T, Zhu Y, Jin Y, Itoh S, Kajiwara M, et al. Clinicalcourse of patients with WASP gene mutations. Blood. 2004;103:456–64.
4. Albert MH, Bittner TC, Nonoyama S, Notarangelo LD, Burns S, Imai K, et al. X-linked thrombocytopenia (XLT) due to WAS mutations: clinical characteristics, long-term outcome, and treatment options. Blood. 2010;115:3231–8.
5. Dupuis-Girod S, Medioni J, Haddad E, Quartier P, Cavazzana-Calvo M, Le Deist F, et al. Autoimmunity in Wiskott-Aldrich syndrome: risk factors, clinical features, and outcome in a single-center cohort of 55 patients. Pediatrics. 2003;111:e622–7.
6. Boyd JK, Cheung CK, Molyneux K, Feehally J, Barratt J. An update on the pathogenesis and treatment of IgA nephropathy. Kidney Int. 2012;81:833–43.
7. Kawasaki Y. Mechanism of onset and exacerbation of chronic glomerulonephritis and its treatment. Pediatr Int. 2011;53:795–806.
8. Moldoveanu Z, Wyatt RJ, Lee JY, Tomana M, Julian BA, Mestecky J, et al. Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. Kidney Int. 2007;71:1148–54.
9. Shimizu M, Nikolov NP, Ueno K, Ohta K, Siegel RM, Yachie A, et al. Development of IgA nephropathy-like glomerulonephritis associated with Wiskott-Aldrich syndrome protein deficiency. Clin Immunol. 2012;142:160–6.
10. Humblet-Baron S, Sather B, Anover S, Becker-Herman S, Kasprowicz DJ, Khim S, et al. Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. J Clin Invest. 2007;117:407–18.
11. Maillard MH, Cotta-de-Almeida V, Takeshima F, Nguyen DD, Michetti P, Nagler C, et al. The Wiskott-Aldrich syndrome protein is required for the function of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells. J Exp Med. 2007;204:381–91.
12. Trifari S, Sitia G, Aiuti A, Scaramuzza S, Marangoni F, Guidotti LG, et al. Defective Th1 cytokine gene transcription in $CD4^{+}$ and $CD8^{+}$ T cells from Wiskott-Aldrich syndrome patients. J Immunol. 2006;177:7451–61.
13. Matsukura H, Kanegane H, Miya K, Ohtsubo K, Higuchi A, Tanizawa T, et al. IgA nephropathy associated with X-linked thrombocytopenia. Am J Kidney Dis. 2004;43:e7–12.
14. Shimizu M, Kanegane H, Wada T, Motoyoshi Y, Morio T, Candotti F, et al. Aberrant glycosylation of IgA in Wiskott-Aldrich syndrome and X-linked thrombocytopenia. J Allergy Clin Immunol. 2013;131:587–90.
15. Sakai O. IgA nephropathy: current concepts and future trends. Nephrology 1997;3: 2–3.
16. Imasawa T, Nagasawa R, Utsunomiya Y, Kawamura T, Zhong Y, Makita N, et al. Bone marrow transplantation attenuates murine IgA nephropathy: role of a stem cell disorder. Kidney Int. 1999;56:1809–17.
17. Suzuki H, Suzuki Y, Aizawa M, Yamanaka T, Kihara M, Pang H, et al. Th1 polarization in murine IgA nephropathy directed by bone marrow-derived cells. Kidney Int. 2007;72:319–27.
18. Inoue T, Sugiyama H, Kikumoto Y, Fukuoka N, Maeshima Y, Hattori H, et al. Downregulation of the beta1, 3- galactosyl transferasegene in tonsillar B lymphocytes and aberrant lectin bindings to tonsillar IgA as a pathogenesis of IgA nephropathy. Contrib Nephrol. 2007;157:120–4.
19. Yamada K, Kobayashi N, Ikeda T, Suzuki Y, Tsuge T, Horikoshi S, et al. Down-regulation of core 1 beta1, 3-galactosyl transferase and Cosmc by Th2 cytokine alters O-glycosylation of IgA1. Nephrol Dial Transplant. 2010;25:3890–7.

20. Suzuki H, Moldoveanu Z, Hall S, Brown R, Vu HL, Novak L, et al. IgA1-secreting cell lines from patients with IgA nephropathy produce aberrantly glycosylated IgA1. J Clin Invest. 2008;118: 629–39.
21. Suzuki H, Suzuki Y, Narita I, Aizawa M, Kihara M, Yamanaka T, et al. Toll-like receptor 9 affects severity of IgA nephropathy. J Am Soc Nephrol. 2008;19:2384–95.
22. Park HJ, Hahn WH, Suh JS, Kim MJ, Kang SW, Lee JS, et al. Association between toll-like receptor 10 (TLR10) gene polymorphisms and childhood IgA nephropathy. Eur J Pediatr. 2011;170: 503–9.
23. Kobayashi I, Nogaki F, Kusano H, Ono T, Miyawaki S, Yoshida H, et al. Interleukin-12 alters the physicochemical characteristics of serum and glomerular IgA and modifies glycosylation in a ddY mouse strain having high IgA levels. Nephrol Dial Transplant. 2002;17:2108–16.
24. Cuevas X, Lloveras J, Mir M, Aubia J, Masramon J. Disappearance of mesangial IgA deposits from the kidneys of two donors after transplantation. Transplant Proc. 1987;19:2208–9.
25. Koselj M, Rott T, Kandus A, Vizjak A, Malovrh M. Donor-transmitted IgA nephropathy: long-term follow-up of kidney donors and recipients. Transplant Proc. 1997;29:3406–7.
26. Ozsahin H, Cavazzana-Calvo M, Notarangelo LD, Schulz A, Thrasher AJ, Mazzolari E, et al. Long-term outcome following hematopoietic stem-cell transplantation in Wiskott-Aldrich syndrome: collaborative study of the European Society for Immunodeficiencies and European Group for Blood and Marrow Transplantation. Blood. 2008;111:439–45.

Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation

Menno C. van Zelm, PhD,[a] Sophinus J. W. Bartol, BSc,[a] Gertjan J. Driessen, MD, PhD,[a,b] Françoise Mascart, MD, PhD,[c] Ismail Reisli, MD,[d] Jose L. Franco, MD,[e] Beata Wolska-Kusnierz, MD,[f] Hirokazu Kanegane, MD, PhD,[g] Louis Boon, PhD,[h] Jacques J. M. van Dongen, MD, PhD,[a] and Mirjam van der Burg, PhD[a] *Rotterdam and Utrecht, The Netherlands, Brussels, Belgium, Konya, Turkey, Medellín, Colombia, Warsaw, Poland, and Toyama, Japan*

Background: Individuals with genetic defects in CD40 ligand (CD40L) or B-cell antigen receptor coreceptor molecules CD19 and CD81 suffer from an antibody deficiency. Still, these patients carry low levels of memory B cells and serum antibodies.

Objective: We sought to assess why the remaining memory B cells and antibodies in the blood of these patients do not provide functional immunity.

Methods: We included CD19-deficient patients (n = 8), CD40L-deficient patients (n = 8), and healthy controls (n = 50) to perform detailed flow cytometry on blood B cells, molecular analysis of IgA and IgG transcripts, as well as functional analysis of B-cell activation.

Results: CD19-deficient and CD40L-deficient patients carried reduced numbers of all memory B-cell subsets except $CD27^-IgA^+$ B cells. Their immunoglobulin heavy chain class-switched transcripts contained less somatic mutations and reduced usage of IgM-distal IgG2 and IgA2 subclasses. The selection strength of mutations for antigen binding was significantly lower than in controls, whereas selection to maintain superantigen binding was normal. Furthermore, the patients showed impaired selection against inherently autoreactive properties of their immunoglobulins. Somatic hypermutation analysis revealed decreased activation-induced cytidine deaminase and uracil-DNA glycosylase 2 activity in CD40L deficiency and increased uracil-DNA glycosylase 2 but decreased mismatch repair in CD19 deficiency. B-cell activation studies revealed that this was at least in part due to transcriptional regulation of DNA repair genes.

Conclusions: This study on CD19 and CD40L deficiencies illustrates that both the B-cell antigen receptor and CD40 signaling pathways are required for the selection of immunoglobulin reactivity. Still, they differentially mediate DNA repair pathways during somatic hypermutation, thereby together shaping the human *in vivo* antigen-experienced B-cell repertoire. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: CD19, CD40L, somatic hypermutation, selection, immunoglobulin, autoreactivity

Circulating B cells each express a unique B-cell antigen receptor (BCR), which has been generated through genomic recombination of their immunoglobulin loci.[1] On recognition of the antigen with their BCR, B cells will initiate an immune response that is characterized by clonal proliferation, and affinity maturation and class switch recombination (CSR) of their immunoglobulin loci. Affinity maturation is accomplished through somatic hypermutation (SHM) in the variable domains of rearranged immunoglobulin heavy (IgH) and immunoglobulin light (Igκ or Igλ) chains, and selection of individual cells for high-affinity antigen binding.[2] CSR changes the effector function of the immunoglobulin molecule without affecting the antigen specificity by replacing the IgH chain constant region.[3]

Both SHM and CSR are genomic processes that are mediated by the activation-induced cytidine deaminase (AID) enzyme (reviewed in Liu and Schatz,[4] Saribasak and Gearhart,[5] and Xu et al[6]). The AID enzyme is upregulated in activated B cells and deaminates cytosines, especially in WRCY/RGYW motifs, in highly transcribed genes. The introduced uracil can be replicated over, giving rise to transition mutations: C→T; or G→A. Alternatively, the uracil can be excised by uracil-DNA glycosylase (UNG), an enzyme involved in the base excision repair (BER) pathway, followed by error-prone repair allowing transversion mutations (C→A/G, G→C/T) of the AID-targeted C·G pair. Finally, the U·G mismatch can be processed by mismatch repair (MMR), which is prone to introduce mutations in A and T residues, preferentially in WA/TW motifs. High-density lesions in immunoglobulin switch regions upstream of the constant genes can result in double-stranded DNA breaks, thereby mediating CSR.

In a classical germinal center (GC) response, the AID enzyme is upregulated through signals from the CD40 molecule following binding with its ligand (CD40L) on activated T follicular helper cells.[7] This interaction is critical for T-cell–dependent immunoglobulin responses: individuals with genetic defects in the

From the Departments of [a]Immunology, and [b]Pediatrics, Erasmus MC, Rotterdam; [c]the Immunobiology Clinic, Hôpital Erasme, and Laboratory of Vaccinology and Mucosal Immunity, Université Libre de Bruxelles, Brussels; [d]the Meram Medical Faculty, Department of Pediatric Immunology and Allergy, Necmettin Erbakan University, Konya; [e]the Group of Primary Immunodeficiencies, University of Antioquia, Medellín, Colombia; [f]the Department of Immunology, Children's Memorial Health Institute, Warsaw; [g]the Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama; and [h]Bioceros B.V., Utrecht.

This work was supported by a grant from Erasmus University Rotterdam (EUR-Fellowship) to M.C.v.Z., and ZonMW Vidi grant 015.008.046 to M.v.d.B.

Disclosure of potential conflict of interest: M. C. van Zelm has received research support from Erasmus University Rotterdam (EUR) in the form of a fellowship, ZonMW, Sophia Children's Hospital Fund, and ACTA Amsterdam. G. J. Driessen has received research support from EUR in the form of a fellowship and has received the Baxter Grant. F. Mascart has received research support from the ECFP-7 program and FRSM. L. Boon has received consultancy fees from Fast Forward Therapeutics; is employed by Bioceros; and has stock/stock options in Fast Forward Therapeutics, Bioceros, Biocerox, and Broteio. M. van der Burg has received research support from ZonMW (Vidi grant no. 91712312). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 5, 2013; revised November 8, 2013; accepted for publication November 8, 2013.

Corresponding author: Menno C. van Zelm, PhD, Department of Immunology, Unit of Molecular Immunology, Erasmus MC, University Medical Center Rotterdam, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands. E-mail: m.vanzelm@erasmusmc.nl.

0091-6749/$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

http://dx.doi.org/10.1016/j.jaci.2013.11.015

Abbreviations used
AID: Activation-induced cytidine deaminase
BCR: B-cell antigen receptor
BER: Base excision repair
CDR: Complementarity determining region
CD40L: CD40 ligand
CSR: Class switch recombination
EXO1: Exonuclease 1
FR: Framework region
GC: Germinal center
IgH: Immunoglobulin heavy chain
MMR: Mismatch repair
MSH2: MutS homolog 2
MSH6: MutS homolog 6
SHM: Somatic hypermutation
SpA: *Staphylococcus aureus* virulence factor protein A
UNG: Uracil-DNA glycosylase

CD40 or CD40L genes lack GC, have strongly reduced serum IgA and IgG levels, and are susceptible to infections with encapsulated bacteria (reviewed in Durandy et al[8]). Still, these patients have IgA^+ plasma cells in gut and IgM^+ and IgA^+ memory B cells in blood carrying SHM.[9-11] Likely candidates for the upregulation of the AID enzyme in the absence of CD40 signaling are TACI and Toll-like receptors that can activate nuclear factor-κB through signaling via MyD88.[12-14]

Developing and mature B cells depend on tonic BCR signaling through PI3K for their survival.[15,16] Antigen-induced BCR signaling depends on CD19, which is complexed with CD21, CD81, and CD225 on the membrane of B cells. Genetic defects in *CD19* or *CD81* that impair CD19 protein expression result in an antibody deficiency with strongly reduced serum immunoglobulin levels.[17-19] Still, naive B-cell survival appears largely normal,[17,20,21] and CD19-deficient patients have normal appearing GC in lymphoid tissue and their BCR-mediated signaling is not completely abolished.[17]

Despite the defects in B-cell activation and the antibody deficiency, patients with mutations resulting in a CD19 deficiency or a CD40L deficiency have memory B cells with molecular signs of immunoglobulin maturation.[9,17-19,22] We here studied memory B cells from these patients as a human *in vivo* model to dissect the roles for BCR and CD40 signaling in immunoglobulin maturation and selection. We found that defective BCR and CD40 signaling both resulted in impaired SHM, CSR, and immunoglobulin repertoire selection, but differentially affected SHM targeting. Thus, these 2 signals are required for optimal antibody responses and selection against inherent autoreactivity.

METHODS

Patients

Eight CD19-deficient and 8 CD40L-deficient patients suffering from an antibody deficiency were included in this study (see Table E1 in this article's Online Repository at www.jacionline.org). Seven CD19-deficient patients had biallelic genetic defects in the *CD19* gene, and 1 patient had a homozygous mutation in the *CD81* gene.[17-19,23,24] Eight male patients suffered from a hyper-IgM syndrome because of the absence of CD40L expression on activated T cells and carried hemizygous mutations in their *CD40L* genes.[9]

Diagnostic workup of blood samples was carried out after oral informed consent and according to the guidelines of the local medical ethics committees in Belgium, Colombia, Japan, Turkey, Poland, and The Netherlands. Controls provided written consent.

Flow cytometry and Ca^{2+} flux analysis

6-Color flowcytometric immunophenotyping of blood B cells was performed for 4 CD19-deficient patients, 5 CD40L-deficient patients, and 50 age-matched healthy controls to quantify 6 memory B cells on a fluorescence-activated cell sorting LSRII.[9]

Ca^{2+} influxes on BCR stimulation were measured in $CD20^+$ B cells by using flow cytometry as described before, following stimulation with *Staphylococcus aureus* Cowan I (Calbiochem, La Jolla, Calif) diluted 1:500, or with 20 μg/mL goat antihuman IgM-F(ab')$_2$ (Jackson Immunoresearch Laboratories, Inc, West Grove, Pa).[17]

Amplification and sequence analysis of rearranged IgH transcripts

IgA and IgG transcripts were amplified from the cDNA of thawed mononuclear cells by using subgroup-specific forward primers in framework region (FR) 1 or the leader sequence of IGHV3 and IGHV4 in combination with a Cα or Cγ consensus reverse primer.[9] The *IGHV1-69* genes that were amplified with the IGHV4 primer were included in all analyses, except for IGHV4 subgroup use. All PCR products were cloned into the pGEM-T easy vector (Promega, Madison, Wis) and prepared for sequencing on an ABI PRISM 3130XL (Applied Biosystems, Foster City, Calif). Obtained sequences were analyzed with the IMGT database (http://www.imgt.org/).[25]

Targeting of SHM in the FR and complementarity determining regions (CDRs) was analyzed with the JoinSolver program.[22] The selection strength for mutations in the FR and the CDR was determined with the Bayesian estimation of Antigen-driven SELectIoN program (http://selection.med.yale.edu/baseline/).[26] This program allows assessment of positive selection of mutations in CDRs as well as negative selection of FR mutations. IgG subclasses and IgA were identified by using the germline sequence of the IGH locus (NG_001019).

In vitro activation of purified B cells from human tonsils

Human tonsils were obtained from otherwise healthy children who underwent routine tonsillectomy. Resting B cells were purified by negative selection with CD43 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and subsequently cultured at a density of 1 million cells/mL in 3 mL culture medium with combinations of IL-4 (10 ng/μL; R&D Systems, Abingdon, United Kingdom), anti-IgM F(ab')$_2$, anti-CD40 (Bioceros B.V., Utrecht, The Netherlands), CpG ODN2006 (1 μg/mL; Miltenyi Biotec), and IL-21 (50 ng/mL; PeproTech, London, United Kingdom). After 72-hour culture, cells were harvested for TaqMan-based quantitative RT-PCR on a StepOnePlus (Applied Biosystems). Intron-spanning primers were designed for each gene by using the universal probe library Assay Design Center (Roche, Basel, Switzerland), and fluorogenic probes were obtained from Roche (see Table E2 in this article's Online Repository at www.jacionline.org). Target gene expression levels were corrected for *ABL* expression levels and expressed as fold difference from uncultured cells.[18] All quantitative RT-PCR reactions were performed in duplicate.

Statistical analyses

Statistical analyses were performed with the Mann-Whitney *U* test, the student paired *t* test, or the χ^2 test as indicated in detail in figure legends. *P* values of less than .05 were considered statistically significant.

RESULTS

Memory B-cell subsets in CD19 and CD40L deficiencies

To study the extent of impaired humoral immunity in CD19-deficient and CD40L-deficient patients (Table E1), we performed extensive flow cytometric immunophenotyping of their blood B-cell compartments. CD40L-deficient patients showed a nearly complete lack of primary ($CD27^+$IgM-only and $CD27^-IgG^+$) and secondary GC-derived ($CD27^+IgG^+$ and $CD27^+IgA^+$) memory B cells, but had clearly detectable $CD27^+IgM^+IgD^+$ "natural effector" B cells and normal numbers of $CD27^-IgA^+$ B cells (see Fig E1 in this article's Online Repository at