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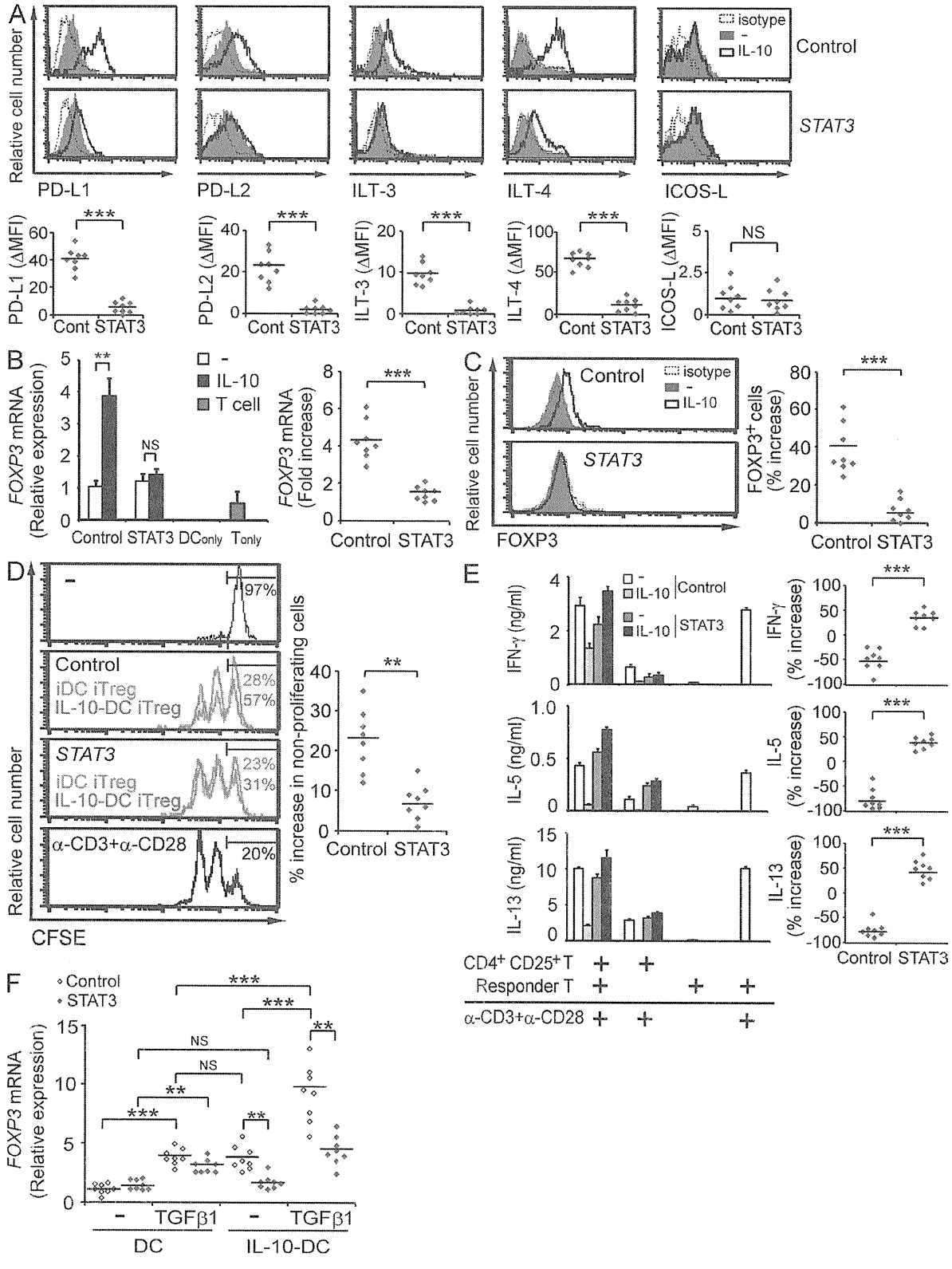

Figure 3. IL-10 signaling defect in MoDCs leads to the defective generation of tolerogenic DCs and iT_{reg} cells. (A) Representative histograms showing the levels of PD-L1, PD-L2, ILT-3, ILT-4, and ICOS-L produced by untreated immature MoDCs (−) and IL-10–DCs (IL-10) from a control subject and a *STAT3* patient are shown at the top. Dashed lines indicate staining with isotype-matched control mAbs. Summary data showing ΔMFI,

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defect to the level of control IL-10–DCs in the presence of TGF-β1 (Fig. 3 F, eighth dataset vs. seventh dataset). To further clarify, we evaluated TGF-β1 and IL-10 production from MoDCs from control subjects and *STAT3* patients (Fig. S6). These results indicate that the production of these inhibitory cytokines from MoDCs is not impaired in *STAT3* patients.

PD-L1, ILT-4, and TGF-β1 in response to IL-10–DCs and STAT3 in DCs play a major role in FOXP3 up-regulation

A recent study in mice demonstrated that PD-L1 plays an important role in inducing FOXP3$^+$ iT$_{reg}$ cells (Keir et al., 2008; Francisco et al., 2009). We investigated whether defective PD-L1 expression in IL-10–DCs from *STAT3* patients played a crucial role in the defective generation of FOXP3$^+$ iT$_{reg}$ cells by adding a peptide neutralizing PD-L1 to the co-culture of IL-10–DCs and naive CD4$^+$ T cells. The addition of this PD-L1 peptide significantly decreased the levels of *FOXP3* mRNA in the naive CD4$^+$ T cells co-cultured with control IL-10–DCs (Fig. 4 A). The addition of the neutralizing peptide had no detectable effect on co-cultures with MoDCs from *STAT3* patients.

We next investigated whether defective ILT-4 expression in IL-10–DCs from *STAT3* patients played an important role in the defective generation of FOXP3$^+$ iT$_{reg}$ cells with a neutralizing mAb to the co-culture of IL-10–DCs and naive CD4$^+$ T cells. The addition of anti–ILT-4 mAb significantly down-regulated the levels of *FOXP3* mRNA in the naive CD4$^+$ T cells co-cultured with control IL-10–DCs compared with a control mAb (Fig. 4 B). The addition of the anti–ILT-4 mAb had no significant effect on the co-culture of the naive CD4$^+$ T cells with patient IL-10–DCs. Thus, in addition to PD-L1, ILT-4 up-regulation in response to IL-10 plays an important role in the generation of FOXP3$^+$ iT$_{reg}$ cells.

We further investigated the contribution of TGF-β1 in the up-regulation of FOXP3 by IL-10–DCs because endogenous TGF-β1 may be supplied by the DCs or from the culture medium. The addition of anti–TGF-β1 mAb significantly down-regulated the levels of FOXP3 mRNA in the naive CD4$^+$ T cells co-cultured with control IL-10–DCs compared with a control mAb (Fig. 4 C). The addition of anti–TGF-β1 mAb had no significant effect on the co-culture of naive CD4$^+$ T cells with patient IL-10–DCs. Thus, TGF-β1 is required for the formation of FOXP3$^+$ iT$_{reg}$ cells in response to control IL-10–DCs.

We also investigated whether DN-STAT3 expression in naive CD4$^+$ T cells plays a significant role in the generation of iT$_{reg}$ cells by evaluating the up-regulation of *FOXP3* mRNA levels in naive CD4$^+$ T cells from *STAT3* patients. The up-regulation of *FOXP3* mRNA levels in response to IL-10–DCs from *STAT3* patients was impaired, but naive CD4$^+$ T cells from control subjects and *STAT3* patients up-regulated *FOXP3* mRNA levels in response to control IL-10–DCs (Fig. 4 D). Thus, DN-STAT3 expression in MoDCs plays a major role in the impairment of *FOXP3* mRNA up-regulation, and DN-STAT3 expression in T cells plays, at most, a minor role in *STAT3* patients.

Primary DCs from *STAT3* patients are defective in IL-10 signaling and up-regulation of PD-L1 and ILT-4

We next investigated the development and function of primary DCs. Two DC subsets were identified in human peripheral blood on the basis of the expression of surface molecules, including CD11c and CD304 (BDCA-4). Lineage marker (Lin) negative HLA-DR$^+$CD11c$^+$CD304$^-$ cells are conventional DCs (cDCs), whereas Lin$^-$HLA-DR$^+$CD11c$^-$CD304$^+$ cells are plasmacytoid DCs (pDCs). The number of PBMCs obtained from the peripheral blood and the percentages of cDCs and pDCs were indistinguishable between control subjects and *STAT3* patients (Fig. 5 A). We next investigated IL-10 signal transduction in primary cDCs and pDCs. The transcriptional up-regulation of *SOCS3* and *VCAN* (*CSPG2*) was impaired in both subsets of primary DCs from *STAT3* patients, as demonstrated by comparison with control subjects (Fig. 5, B and C). We evaluated the effect of prior treatment with IL-10 on the phenotypic maturation of primary cDCs. IL-10 was added to the culture 1 d before LPS treatment, which inhibited the LPS-induced maturation by inhibiting the up-regulation of CD83 and CD86 in control subjects.

IL-10–treated minus untreated, of PD-L1, PD-L2, ILT-3, ILT-4, and ICOS-L (n = 8 each) are at the bottom. (B) Q-PCR analysis of *FOXP3* mRNA levels after the co-culture of third-party allogeneic naive CD4$^+$ T cells from a control subject with untreated immature MoDCs (−) or IL-10–DCs (IL-10) from a control subject and a *STAT3* patient. Cultures in the absence of naive CD4$^+$ T cells (DC only) and MoDCs (T only) were used as negative controls. Representative data are on the left, and summary data (n = 8 each) showing fold increase are on the right. (C) Flow cytometric analysis of cytoplasmic FOXP3 protein levels in naive CD4$^+$ T cells co-cultured with untreated immature MoDCs (−) and IL-10–DCs (IL-10) from a control subject and a *STAT3* patient. Staining with isotype-matched control mAbs is indicated by dashed lines. Representative data are on the left, and summary data (n = 8 each) showing percent increase are on the right. (D) CFSE-labeled CD4$^+$CD25$^-$ responder T cells were cultured alone in the absence (−) or presence of anti-CD3 and anti-CD28 mAbs or with iT$_{reg}$ cells generated by co-culture with control or *STAT3* patient immature DCs (iDCs) or IL-10–DCs. After 5 d, the proliferation of CFSE-labeled responder T cells was assessed by flow cytometry. Representative histograms are on the left, and summary data (n = 8 each) showing the percent increase in nonproliferating cells, numbers in magenta minus numbers in blue, are on the right. (E) Cytokine levels in the supernatants of co-cultures of responder T cells and iT$_{reg}$ cells, as indicated. Representative data are on the left, and summary data (n = 8 each) showing percent increase are on the right. Data are representative of at least two independent experiments. (F) Q-PCR analysis of *FOXP3* mRNA expression after the co-culture of third-party allogeneic naive CD4$^+$ T cells from a control subject with untreated immature MoDCs (−) or IL-10–DCs from a control subject and a *STAT3* patient in the absence or presence of exogenous TGF-β1. We show summary data showing relative *FOXP3* expression (n = 8 each) performed in triplicate. Data are representative of at least two independent experiments. (B and E) Graphs show mean ± SD. (A–F) Horizontal bars indicate mean values. **, $P < 0.01$; ***, $P < 0.001$.

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In contrast, the maturation of primary cDCs derived from *STAT3* patients was almost intact by prior treatment with IL-10 (Fig. 5 D). We did not detect inhibitory effects of IL-10 on CD80 up-regulation in control subjects and *STAT3* patients. Furthermore, control primary cDCs up-regulated the expression of inhibitory molecules, including PD-L1, PD-L2, ILT-3 (unpublished data), and ILT-4 by IL-10 treatment. The up-regulation of these inhibitory molecules was impaired in the primary cDCs of *STAT3* patients (Fig. 5 E). These results demonstrate that IL-10 signaling is defective not only in MoDCs but also in primary DCs, resulting in the defective up-regulation of surface inhibitory molecules in *STAT3* patients.

TYK2-deficient MoDCs are also defective in the generation of tolerogenic DCs and iT_{reg} cells

We studied MoDCs from a patient with *TYK2* deficiency to confirm that the IL-10 signaling defect was responsible for the defective generation of tolerogenic DCs and iT_{reg} cells. The absolute numbers of cDCs and pDCs in PBMCs were similar in the *TYK2*-deficient patient and a control subject, and no significant difference in the differentiation of MoDCs was observed on evaluations of forward and side light scatter, CD1a expression, and the expression of CD80, CD83, and CD86 of DCs before and after LPS-induced maturation (Fig. S7, A–D). No inhibition of the up-regulation of CD80, CD83, and CD86 by prior treatment with IL-10 was detectable in cells from the *TYK2*-deficient patient (Fig. 6 A). The up-regulation of PD-L1, PD-L2, ILT-3, and ILT-4 on MoDCs was also defective in the *TYK2*-deficient patient, as shown by comparisons with control subjects (Fig. 6 B). An increase in *FOXP3* mRNA and protein levels was detectable in co-cultures of allogeneic naive $CD4^+$ T cells with control IL-10–DCs but not in co-cultures with *TYK2*-deficient IL-10–DCs (Fig. 6, C and D). Consistent with these observations, no suppression of naive $CD4^+$ T cell proliferation and cytokine production (including IFN-γ, IL-5, and IL-13) was detected when *TYK2*-deficient IL-10–DCs were used

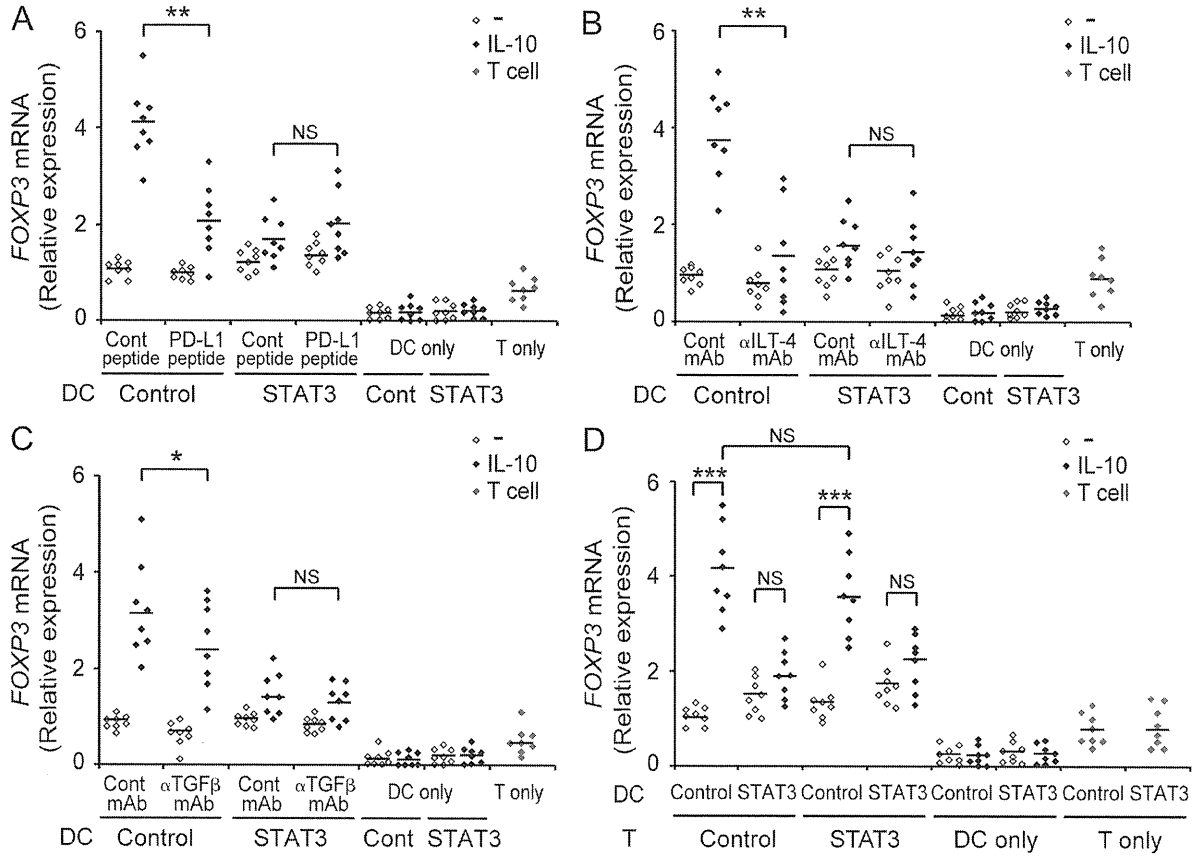

Figure 4. PD-L1, ILT-4, and TGF-β1 in response to IL-10–DCs and *STAT3* in DCs play a major role in FOXP3 up-regulation. (A–C) Q-PCR analysis of *FOXP3* mRNA levels in third-party allogeneic naive $CD4^+$ T cells from control (Cont) subjects co-cultured with untreated immature MoDCs (−) and IL-10–DCs (IL-10) from eight control subjects and eight *STAT3* patients. A neutralizing PD-L1 peptide or a control peptide (A), control or ILT-4–neutralizing mAb (B), or control or TGF-β–neutralizing mAb (C) was added where indicated. (D) Q-PCR analysis of *FOXP3* mRNA levels in third-party allogeneic naive $CD4^+$ T cells from control subjects and *STAT3* patients co-cultured with untreated immature DCs (−) or IL-10–DCs (IL-10) from control subjects and *STAT3* patients. Summary data show relative *FOXP3* mRNA expression ($n = 8$ each) and were performed in triplicate. Data are representative of at least two independent experiments. (A–D) Horizontal bars indicate mean values. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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(Fig. 6, E and F). MoDCs from the *TYK2*-deficient patient produced an equivalent amount of TGF-β1 and reduced amount of IL-10 compared with a control subject, which might be associated with the fact that the type I IFN signal is impaired in the *TYK2*-deficient patient but not in *STAT3* patients. (Fig. S7, E and F). Thus, the IL-10 signaling defect in HIES patients, *STAT3* patients, and the *TYK2*-deficient patient results in the impaired generation of tolerogenic DCs and iT_{reg} cells.

DISCUSSION

We found that the Th1 and Th2 differentiation of naive $CD4^+$ T cells and the suppressive activity of T_{reg} cells were normal in *STAT3* patients. Recent data have shown that Ig isotype switching in B cells is normal in *STAT3* patients (Avery et al., 2010). Thus, it is not likely that T cell– and B cell–intrinsic mechanisms are responsible for the allergic manifestations in HIES patients. We then investigated DCs, which can regulate the immune response and tolerance. IL-10 signal transduction was defective in the primary DCs and MoDCs of patients, despite the intact TGF-β1 signal transduction in these cells. This defect resulted in impairment of the suppression of cytokine production and T cell proliferation by IL-10–DCs. The generation and suppressive activity of $FOXP3^+$ iT_{reg} cells cultured with IL-10–DCs was impaired in HIES patients. The defective generation of tolerogenic DCs and iT_{reg} cells in response to IL-10 was also observed in the other type of HIES, *TYK2* deficiency. These results suggest that IL-10 signaling in DCs may be crucial for the generation of tolerogenic DCs and iT_{reg} cells to maintain an appropriate Th1–Th2–T_{reg} cell balance in HIES patients.

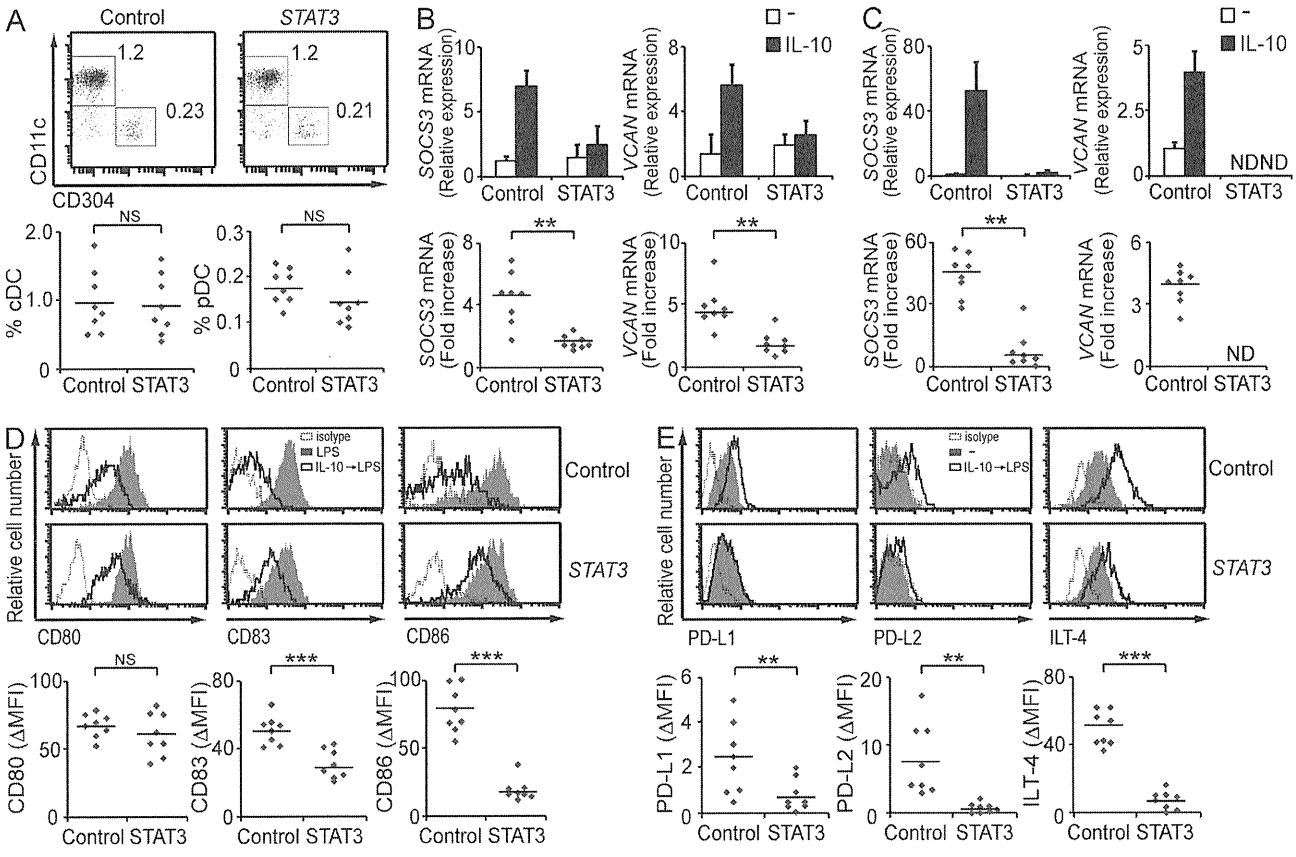

Figure 5. Primary DCs are defective in IL-10 signal and up-regulation of PD-L1 and ILT-4. (A) Dot blots are gated on Lin-negative HLA-DR-positive cells from a control subject and a *STAT3* patient. cDCs are $CD11c^+CD304^-$ (BDCA-4), and pDCs are $CD11c^-CD304^+$. Representative dot plots from a control subject and a *STAT3* patient are shown at the top, and pooled data ($n = 8$ each) showing percentages of cDCs and pDCs are at the bottom. (B and C) Primary cDCs (B) and pDCs (C) from a control subject and a *STAT3* patient were stimulated with IL-10 for 2 h, and the amounts of *SOCS3* and *VCAN* (*CSPG2*) mRNAs were analyzed by Q-PCR. Representative data are shown at the top, normalized to *HPRT* levels, with the level of unstimulated control cells defined as 1.0. Summary data ($n = 8$ each) showing fold increase are at the bottom. Data are representative of at least two independent experiments performed in triplicate. Graphs show mean ± SD. (D) Representative histograms of CD80, CD83, and CD86 expression on control and *STAT3* cDCs stimulated with LPS alone or LPS after IL-10 treatment. Dashed lines indicate staining with isotype-matched control mAbs. Summary data showing ΔMFI, LPS stimulated minus LPS-stimulated IL-10–DCs, ($n = 8$ each) are at the bottom. (E) Representative histograms of PD-L1, PD-L2, and ILT-4 expression of primary cDCs (−) and IL-10–treated (IL-10) primary cDCs from a control subject and a *STAT3* patient are shown at the top. Summary data ($n = 8$ each) showing ΔMFI, IL-10 treated minus untreated, of PD-L1, PD-L2, and ILT-4 are shown at the bottom. Data are representative of at least two independent experiments. (A–E) Horizontal bars indicate mean values. **, $P < 0.01$; ***, $P < 0.001$.

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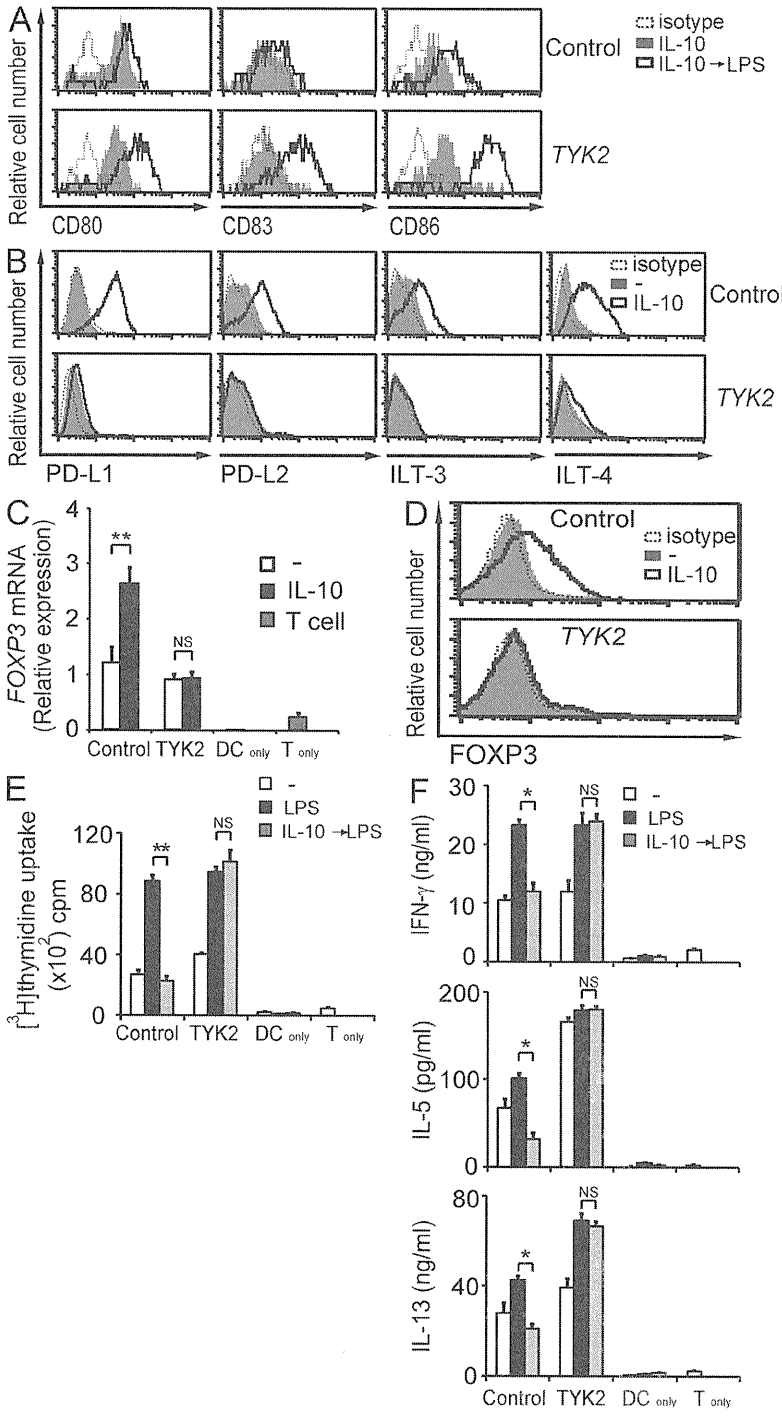

Figure 6. *TYK2*-deficient MoDCs display defective generation of tolerogenic DCs and iT_{reg} cells. (A) Flow cytometric analysis of the CD80, CD83, and CD86 expression on IL-10–DCs (IL-10) and LPS-matured MoDCs after prior treatment with IL-10 (IL-10 → LPS), with cells obtained from a control subject and a patient with *TYK2* deficiency. We show representative histograms from three independent experiments. (B) Flow cytometric analysis of the levels of PD-L1, PD-L2, ILT-3, and ILT-4 in untreated immature MoDCs (−) and IL-10–DCs (IL-10) from a control subject and a *TYK2* deficiency. We show representative histograms from three independent experiments. (C) Q-PCR analysis of *FOXP3* mRNA levels after the co-culture of third-party allogeneic naive $CD4^+$ T cells from a control subject with untreated immature MoDCs (−) or IL-10–DCs (IL-10) from a control subject and a *TYK2*-deficient patient. We show representative data from three independent experiments. (D) Flow cytometric analysis of cytoplasmic FOXP3 protein levels in untreated immature MoDCs (−) and IL-10–DCs (IL-10) from a control subject and a *TYK2*-deficient patient. Isotype-matched control (isotype) antibody staining is indicated by a dashed line. We show representative histograms from three independent experiments. (E) Third-party allogeneic naive $CD4^+$ T cells from control subjects were co-cultured with immature MoDCs (−), LPS-matured MoDCs (LPS), or LPS-matured MoDCs after prior treatment with IL-10 (IL-10 → LPS). The cells were obtained from a control subject and a *TYK2*-deficient patient. After 5 d, proliferation was evaluated by pulsing the cells with 1 µCi (37 kBq) [^{3}H]thymidine for the final 18 h of culture. All samples were evaluated in triplicate. We show representative data from three independent experiments. (F) Cells were cultured as in E, and cytokine levels were evaluated as indicated. All samples were evaluated in triplicate. We show representative data from at least three independent experiments. (C, E, and F) Graphs show mean ± SD. *, $P < 0.05$; **, $P < 0.01$.

The exposure of the skin to allergens induces allergen-specific unresponsiveness, possibly because of the production of IL-10 by keratinocytes (Enk and Katz, 1992; Enk et al., 1993). Langerhans cells and dermal DCs receive IL-10 signal and induce allergen-specific tolerance. This defect in IL-10–mediated tolerance to innocuous environmental antigens may be one of the mechanisms underlying the allergic signs in HIES patients. In humans, we are not certain about the nT_{reg} cell/iT_{reg} cell ratio in the peripheral blood under resting conditions. Our data suggest that most of the T_{reg} cells in the peripheral blood are nT_{reg} cells, which are derived from the thymus and are independent of the IL-10 signal. iT_{reg} cells in the peripheral blood may be a minor population under resting conditions but may play a crucial role in the regulation of antigen-specific allergic reactions.

Human peripheral blood T_{reg} cells suppressed proliferation and Th2 cytokine production by responder T cells stimulated with allergens (Bellinghausen et al., 2003; Grindebacke et al., 2004; Ling et al., 2004). $CD4^+$ T cells cultured with IL-10–DCs have antigen-specific iT_{reg} cell activity (Steinbrink et al., 2002). In vitro experiments suggested that the suppression is dependent on cell to cell contact between iT_{reg} cells and responder T cells and is not mediated by soluble factors. In this study, we found that the generation of $FOXP3^+$ iT_{reg} cells by

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IL-10–DCs was impaired in HIES patients. Evidence is accumulating to suggest that interactions between tolerogenic DCs and T_{reg} cells play an important role in the maintenance of immune tolerance against self-antigens and innocuous environmental antigens (Yamazaki et al., 2006a; Hubert et al., 2007). $CD4^+CD25^+$ T_{reg} cell populations can expand in the presence of DCs with intact suppressive activity in vitro and in vivo (Yamazaki et al., 2006b). In addition to the IL-10 signal provided by the cells sensing innocuous environmental antigens, the IL-10–mediated positive feedback loop between tolerogenic DCs and iT_{reg} cells is probably impaired in HIES patients, and this may also constitute one of the mechanisms underlying the atopic signs in HIES patients.

A large number of clinical studies have demonstrated that IL-10 is involved in the molecular pathogenesis of atopic disorders in humans. The frequency of allergen-specific, IL-10–secreting T cells is significantly higher in nonatopic individuals than in atopic patients (Akdis et al., 2004). IL-10 levels are inversely correlated with the severity of human allergic diseases (Borish et al., 1996; Lim et al., 1998). Furthermore, allergen-specific immunotherapies increase IL-10 synthesis by T cells (Francis et al., 2003; Vissers et al., 2004). All of these findings suggest that IL-10 plays a key role in the control of atopic diseases in humans.

In contrast, mice lacking IL-10 or the IL-10 receptor develop spontaneous inflammation in the large intestine (Kühn et al., 1993; Davidson et al., 1996; Spencer et al., 1998). Mice with a T_{reg} cell–specific IL-10 deficiency also display inflammation of surfaces in contact with the environment such as the colon, lungs, and skin (Rubtsov et al., 2008). In humans, mutations in the genes encoding IL-10 receptor subunits have been found in patients with early-onset enterocolitis (Glocker et al., 2009). Thus, a lack of IL-10 signaling results in enterocolitis in both humans and mice. Interestingly, in patients with HIES, immune responses to innocuous environmental antigens are limited to the skin, with no marked increase in the frequency of enterocolitis. One possible reason for this discrepancy is the existence of a partial, as opposed to complete, IL-10 signaling deficiency in *STAT3* patients, creating a situation resembling T_{reg} cell–specific IL-10 deficiency. An alternative nonmutually exclusive explanation is that, in addition to the IL-10 signaling defect, *STAT3* patients have defective Th17 cell development (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008; Minegishi et al., 2009). The combination of Th17 cell deficiency and IL-10 signaling may result in allergic signs but prevent the development of enterocolitis (Brand, 2009).

T_{reg} cells mediate peripheral tolerance and play a central role in determining several immunopathologies, including autoimmunity, chronic infections, tumor development, and allergies (Hawrylowicz and O'Garra, 2005). $FOXP3^+$ T_{reg} cells are involved in protecting humans against allergic diseases, as patients with IPEX syndrome suffer from allergic symptoms (Bennett et al., 2001; Wildin et al., 2001). PBMCs from atopic patients proliferate more extensively and produce more Th2 cytokines in response to allergens than do PBMCs from nonatopic healthy individuals (Taams et al., 2002; Ling et al., 2004). However, patients with atopic dermatitis have normal numbers of T_{reg} cells in the periphery with normal suppressive activity (Ou et al., 2004). These results suggest that iT_{reg} cells may be more important than nT_{reg} cells in controlling atopic dermatitis. Consistent with this hypothesis, a recent study using two mouse strains, one capable of generating iT_{reg} cells but incapable of generating nT_{reg} cells and the other unable to generate either iT_{reg} or nT_{reg} cells, suggested that iT_{reg} cells controlled allergic inflammation against innocuous environmental allergens, whereas nT_{reg} cells did not (Curotto de Lafaille et al., 2008).

TGF-β1 is the other crucial inhibitory cytokine regulating lymphocyte homeostasis, inhibiting Th1 and Th2 cell responses and promoting the differentiation of iT_{reg} cells (Li et al., 2006). One previous study suggested that STAT3 might be involved in transduction of the TGF-β1 signal (Ohkawara et al., 2004), but we detected no impairment of TGF-β1 signaling in DCs from *STAT3* patients. Unexpectedly, we found that TGF-β1 and IL-10–DCs operated synergistically to up-regulate FOXP3 expression in naive $CD4^+$ T cells. This suggests that the defective generation of IL-10–DCs may have a far-reaching impact on the induction of iT_{reg} cells in HIES patients.

We provide in this study the first demonstration that an IL-10 signaling defect leads to the impairment of tolerogenic DC and iT_{reg} cell production in the HIES. These results suggest that the defect in tolerogenic DC and iT_{reg} cell production, even in the presence of normal nT_{reg} cells, may contribute to the development of complex clinical manifestations, including allergic inflammation in HIES patients. Furthermore, a unique combination of defective Th17 differentiation and iT_{reg} cell generation may culminate in the development of atopic dermatitis but not enterocolitis in HIES patients.

MATERIALS AND METHODS

Patients. All *STAT3* patients enrolled in this study had typical clinical findings associated with HIES and a National Institutes of Health score >40 points (Table I; Grimbacher et al., 1999). The diagnosis was confirmed by the identification of mutations in the *STAT3* gene. The patient with *TYK2* deficiency has been described elsewhere (Minegishi et al., 2006). The study was approved by the Tokyo Medical and Dental University Ethics Committee, and written informed consent was obtained from all patients. Control individuals were nonatopic, age-matched, and equivalent in sex distribution to HIES patients. All of the patients and control subjects were in a healthy state when their blood samples were collected.

Antibodies, cytokines, and peptides. We used mAbs against CD4 (RPA-T4), CD14 (M5E2), CD11c (B-ly6), CD123 (9F5), HLA-DR (TU36), CD25 (M-A251), CD62L (Dreg 56), CD1a, (HI149), CD80 (L307.4), CD86 (2331), CD83 (HB15e), PD-L1 (MIH1), PD-L2 (MIH18), FOXP3 (259D/C7), and CTLA-4 (CD152; BNI3), a Lin cocktail (antibodies against CD3 [SK7], CD14 [MΦP9], CD16 [3G8], CD19 [SJ25C1], CD20 [L27], and CD56 [NCAM16.2]), and mAbs against IFN-γ (4S.B3) and IL-4 (8D4-8), neutralizing mAbs against IFN-γ (B27), IL-4 (MP4-25D2), and isotype-matched control mAbs, all of which were purchased from BD. We obtained antibodies against ILT-3 (CD85K; 293623), ILT-4 (CD85d; 287219), LAP (latency-associated peptide; TGF-β1; 27235), and GITR (TNFRSF18; 110416) from R&D Systems. Anti–ICOS-L antibody (MIH12) was obtained from eBioscience. Anti-CD304 (BDCA-4) antibody (AD5-17F6) was obtained

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Table I. Characteristics of HIES patients

Patient	Age	Sex	Mutation	Domain	Highest IgE	NIH score															
						Total points	Skin abscess	Pneumonia	Eosinophilia	Newborn rash	Eczema	URI	Candidiasis	Serious infection	Lung abnormality	Face	Nasal width	Retained teeth	Scoliosis	Fracture	Hyperextensibility	High palate
	yr				*IU/ml*																
STAT3-1	23	F	ΔV463	DNA binding	17,500	58	2	8	0	4	2	0	0	0	8	5	3	8	4	0	4	0
STAT3-2	11	F	R382W	DNA binding	97,900	66	8	8	6	4	4	2	1	0	8	2	1	8	0	0	4	0
STAT3-3	24	M	ΔV463	DNA binding	62,000	56	8	0	6	4	4	0	4	4	8	5	1	0	0	0	0	2
STAT3-4	13	M	R382Q	DNA binding	11,600	41	8	8	0	4	4	0	0	4	0	2	1	0	0	0	0	0
STAT3-5	16	F	H437Y	DNA binding	50,600	44	8	4	6	4	4	0	0	0	0	5	3	0	0	0	0	0
STAT3-6	23	F	S636F	SH2	25,400	68	8	8	0	4	2	2	4	0	6	5	3	8	0	4	4	0
STAT3-7	49	F	G618D	SH2	21,300	53	8	8	0	4	4	0	0	4	8	2	1	0	0	4	0	0
STAT3-8	34	M	Δ371-380	DNA binding	12,300	53	8	0	0	4	4	0	4	4	0	5	3	1	0	4	4	2
TYK2-1	23	M	Frame shift	NA	2,100	48	8	8	3	4	4	2	1	8	0	0	0	0	0	0	0	0

NA, not applicable. Possible HIES patients are evaluated by the National Institutes of Health (NIH) scoring system. If the total points of NIH score are >40 points, the patient is considered as HIES clinically. NIH score is defined as follows. If the highest serum IgE level is >2,000 IU/ml, the patient scores 10 points. Skin abscess: 8 points indicate more than four, and 2 points indicate one or two episodes of skin abscess in lifetime. Pneumonia: 8 points indicate more than three, and 4 points indicate two episodes of pneumonia in lifetime. Eosinophilia: 6 points indicate >800 eosinophils/μl, and 3 points indicate 700–800 eosinophils/μl of blood (700/μl = 1 SD and 800/μl = 2 SD above the mean value from normal individuals). Newborn rash: 4 points indicate newborn rash is present. Eczema: 4 points indicate eczema is severe, and 2 points indicate eczema is moderate in worst stage. Upper respiratory infections (URI): 2 points indicate the patient sufferers from upper respiratory infections six to four times, 1 point indicates three times per year. Candidiasis: 4 points indicate the patient has systemic candidiasis, and 1 point indicates oral candidiasis. Serious infections: 8 points indicate the patient has episodes of fatal and serious infection, and 4 points indicate the patient has an episode of serious infection. Lung abnormality: 8 points indicate the patient has pneumatocele, and 6 points indicate the patient has bronchiectasis. Face: 5 points indicate the patient has typical characteristic facial appearance, and 2 points indicate mild characteristic facial appearance. Nasal width: 3 points indicate the patient has nasal width of >2 SD, and 1 point indicates nasal width with 1–2 SD. Retained teeth: 8 points indicate the patient has more than three retained primary teeth, and 1 point indicates the patient has one retained primary tooth. Scoliosis: 4 points indicate the patient has scoliosis of 15–20°, and 2 points indicate scoliosis of 10–14°. Fracture: 4 points indicate the patient has one or two episodes of fracture with minor trauma. Hyperextensibility: 4 points indicate the patient has hyperextensible joints. High palate: 2 points indicate the patient has a high palate. In all items, 0 points indicate the finding is absent. None of the patients have lymphoma or midline anomaly.

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from Miltenyi Biotec. Recombinant human (rh) GM-CSF, IL-4, IFN-γ, IL-10, and TGF-β1 were purchased from PeproTech. Neutralizing PD-L1 peptide was obtained from Abcam, and an irrelevant peptide was used as a negative control.

PBMCs and naive CD4$^+$ T cell culture. PBMCs were isolated by Ficoll density gradient centrifugation (Histopaque-1077; Sigma-Aldrich). PBMCs were cultured in 96-well plates in RPMI 1640 medium supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 100 mM sodium pyruvate, nonessential amino acids, minimal essential medium vitamins (all from Invitrogen), 50 U/50 μg/ml penicillin/streptomycin (Nacalai Tesque), and 50 μM mercaptoethanol. Cultures were stimulated with a 1:100 (vol/vol) dilution of anti-CD3/CD28 mAb–coated beads from Invitrogen. For some experiments, the following mAbs and cytokines were added: 10 ng/ml rhIFN-γ, 10 ng/ml rhIL-4, and neutralizing antibodies against 10 μg/ml IFN-γ and 10 μg/ml IL-4.

T_{reg} cell purification and functional assay. Total CD4$^+$ T cells were isolated with the CD4$^+$ T cell isolation kit (BD). The cells were stained for sorting with antibodies against CD4, CD25, and CD62L. All mAbs were used after dialysis to remove sodium azide (Baecher-Allan et al., 2006). CD4$^+$CD25$^-$CD62L^{hi} responder T cells and CD4$^+$CD25$^+$CD62L^{hi} T_{reg} cell populations were isolated by sorting with a cell sorter (Moflo; Beckman Coulter). In the postsort analysis, the resulting cell preparation was found to be to >99% purity. Co-culture was set up as follows: 1.25×10^4 responder T cells and 1.25×10^3 T_{reg} cells were co-cultured for 5 d with a 1:100 (vol/vol) dilution of magnetic beads coated with antibodies against CD3/CD28. Responder T cells were used as a negative control. Proliferation was assessed by adding 1 μCi (37 kBq) [^{3}H]thymidine (methyl-[^{3}H]thymidine; ICN Biomedicals) to the culture medium for the final 18 h.

Isolation of primary DCs. Primary DCs were obtained by the enrichment using a human DC enrichment set (BD) and cell sorting with FACS Aria II (BD): cDCs as Lin$^-$HLA-DR$^+$ CD11c$^+$CD304$^-$ cells and pDCs as Lin$^-$HLA-DR$^+$CD11c$^-$CD304$^+$ cells. In the postsort analysis, the resulting cell preparation was to >99% purity.

In vitro generation of MoDCs. CD14$^+$ monocytes were isolated from PBMCs with immunomagnetic beads (BD) at a purity of >98%. Monocytes were cultured in the presence of 50 ng/ml GM-CSF and 10 ng/ml IL-4 for 5 d. For differentiation into mature DCs, immature DCs were stimulated on day 5 with 100 ng/ml LPS (O55:B5; Sigma-Aldrich). For the generation of tolerogenic DCs, 100 ng/ml IL-10 was added to the culture on day 3. Nonadherent DCs on day 7 were used for T cell stimulation.

Allogeneic naive CD4$^+$ T cell proliferation assay. Naive CD4$^+$ T cells were negatively selected from PBMCs through the depletion of CD8, CD11b, CD16, CD19, CD36, CD41a, CD45RO, CD56, CD123, γδ-TCR, and glycophorin A–positive cells, with antibody-coated paramagnetic microbeads (naive CD4$^+$ T cell isolation kit from BD), according to the manufacturer's protocol. The purity of the naive CD4$^+$ T cell preparation exceeded 95%. For proliferation assays, 10^5 naive CD4$^+$ T cells were co-cultured in 96-well round-bottomed plates, in triplicate, with 10^4 allogeneic DCs. After 5 d, the cells were pulsed with 1 μCi (37 kBq) per well of [^{3}H]thymidine for 18 h, and [^{3}H]thymidine incorporation was evaluated with a β counter (model 1450; PerkinElmer).

iT_{reg} cell preparation and functional evaluation. Naive CD4$^+$ T cells were obtained from PBMCs with the naive CD4$^+$ T cell isolation kit. We obtained CD4$^+$CD25$^-$ responder T cells by depleting the CD25$^+$ cells with magnetic beads coated with an antibody against CD25 (BD). The resulting cell preparation was >95% pure. We obtained iT_{reg} cells by setting up cocultures as described for the Allogeneic naive CD4$^+$ T cell proliferation assay and purifying CD4$^+$CD25$^+$ cells after 3 d with immunomagnetic beads. CD4$^+$CD25$^+$ iT_{reg} cells were co-cultured with CFSE-labeled autologous CD4$^+$CD25$^-$ responder T cells in 96-well round-bottomed plates containing a 1:100 (vol/vol) dilution of anti-CD3/CD28 mAb beads. After 5 d, the proliferation of the CFSE-labeled CD4$^+$CD25$^-$ T cells was assessed by flow cytometry.

Flow cytometric analysis. Cells were analyzed on a FACSCalibur or FACSCanto II machine (BD) using CellQuest or FACSDiva software (BD).

Mannose receptor–mediated endocytosis. 1 mg/ml FITC–dextran (Sigma-Aldrich) was incubated with 10^5 cells at 37°C or 4°C for 2 h. FITC-dextran uptake was stopped by adding ice-cold PBS, and the cells were then thoroughly washed in a refrigerated centrifuge. Samples were then subjected to flow cytometry. The level of antigen uptake by DCs was assessed as the difference between the test (37°C) and control (4°C) values for each sample.

Cytokine ELISA. For cytokine determinations, the culture supernatant was stored at −80°C until use, and the amounts of IFN-γ, TNF, IL-5, IL-6, IL-10, IL-12p40, and IL-13 present were then determined by ELISA, according to the kit manufacturer's instructions (BD).

Intracellular staining. Naive CD4$^+$ T cells were cultured with plate-bound antibodies against CD3 and CD28 in Th1 conditions, IFN-γ plus antibody against IL-4 in Th2 conditions, or IL-4 and antibody against IFN-γ, and the cells were then fixed and permeabilized (Cytofix/Cytoperm reagents; BD) and stained with mAbs against CD4, IFN-γ, and IL-4, according to the manufacturer's instructions (BD). CTLA-4 staining was performed after Cytofix/Cytoperm treatment.

FOXP3 intracellular staining. Naive CD4$^+$ T cells co-cultured with untreated DCs or IL-10–DCs were fixed and permeabilized with the human FOXP3 buffer set (BD) and stained with mAb against FOXP3.

RNA isolation and real-time quantitative RT-PCR (Q-PCR). Cells were harvested for total RNA isolation with the Fastpure RNA kit (Takara Bio Inc.). Total RNA was reverse transcribed with Primescript RT (Takara Bio Inc.). An aliquot of the RT products was used as a template for real-time PCR with SYBR green Mastermix (Takara Bio Inc.) on an Mx3005P thermocycler (Agilent Technologies) with SYBR green I dye as the amplicon detector and ROX as the passive reference. The gene for HPRT (hypoxanthine phosphoribosyltransferase) was amplified as an endogenous reference. Quantification was achieved by both the standard curve and comparative ΔΔCT methods.

Data analysis. Data are expressed as means ± the SD. Unpaired *t* tests or analysis of variance was used for statistical analysis. P-values <0.05 were considered significant (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$).

Online supplemental material. Fig. S1 shows normal Th1 and Th2 differentiation from naive CD4$^+$ T cells but increased Th2 cytokine production from activated T cells in PBMCs of *STAT3* patients. Fig. S2 shows that MoDC differentiation in vitro and TGF-β1 signaling in MoDCs are intact in *STAT3* patients. Fig. S3 shows that IL-10 treatment does not impair the differentiation of MoDCs, but down-regulation of CD80, CD83, and CD86 is defective in MoDCs from *STAT3* patients. Fig. S4 shows that suppression of proliferation by IL-10 pretreatment is impaired in MoDCs from *STAT3* patients. Fig. S5 shows that up-regulation of FOXP3, CTLA-4, and GITR is impaired in iT_{reg} cells co-cultured with patient IL-10–DCs. Fig. S6 shows that MoDCs from *STAT3* patients produce equivalent amounts of TGF-β1. Fig. S7 shows the characterization of primary DCs and MoDCs from the patient with *TYK2* deficiency. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100799/DC1.

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Nationwide Survey of Patients with Primary Immunodeficiency Diseases in Japan

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Abstract To determine the prevalence and clinical characteristics of patients with in Japan, we conducted a nationwide survey of primary immunodeficiency disease (PID) patients for the first time in 30 years. Questionnaires were sent to 1,224 pediatric departments and 1,670 internal medicine departments of Japanese hospitals. A total of 1,240 patients were registered. The estimated number of patients with PID was 2,900 with a prevalence of 2.3 per 100,000 people and homogenous regional distribution in Japan. The male-to-female ratio was 2.3:1 with a median age of 12.8 years. Adolescents or adults constituted 42.8% of the patients. A number of 25 (2.7%) and 78 (8.5%) patients developed malignant disorders and immune-related diseases, respectively, as complications of primary immunodeficiency disease. Close monitoring and appropriate management for these complications in addition to prevention of infectious diseases is important for improving the quality of life of PID patients.

Keywords Primary immunodeficiency disease · epidemiology · nationwide survey · Japan

Abbreviations

APECED	Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy
BTK	Bruton's tyrosine kinase
CGD	Chronic granulomatous disease
CID	Combined T and B cell immunodeficiency
CVID	Common variable immunodeficiency disease
FMF	Familial Mediterranean fever
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked
NEMO	Nuclear factor kappa B essential modulator
PID	Primary immunodeficiency disease
SIgAD	Selective IgA deficiency
SLE	Systemic lupus erythematosus

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TRAPS	Tumor necrosis factor receptor-associated periodic syndrome
WAS	Wiskott–Aldrich syndrome
WHIM	Warts hypogammaglobulinemia, infections, and myelokathexis

Introduction

Patients with primary immunodeficiency disease (PID) show susceptibility to infections due to congenital immune system defects. These patients are also associated with noninfectious complications including autoimmune diseases and malignant disorders. Recent studies have revealed the causes of many PIDs to be mutations in various genes encoding molecules involved in the host defense mechanisms [1]. In addition, various new PIDs including defects in innate immunity and autoinflammatory disorders were identified under the recent progress in immunology and molecular genetics [2]. PID classification has been revised according to the identification of new PIDs and on the basis of new findings in PID pathophysiology. For a more precise clinical analysis, data should be obtained in accordance with the latest PID classifications.

The first nationwide survey of patients with PID in Japan was conducted between 1974 and 1979, which included 497 registered cases [3]. By 2007, a total of 1,297 patients were cataloged by a small number of PID specialists into a registration system [4]. The approximate prevalence of PID patients in Japan in the first nationwide survey was 1.0 in 100,000 people, which was much lower than that in other countries [5–7]. This difference in PID prevalence between Japan and other countries suggested that some PID patients in Japan remained unregistered. To determine the prevalence and clinical characteristics of patients with PID in Japan on the basis of the recent international classification system for PID, we conducted a nationwide survey of PID for the first time in 30 years.

Methods

This study was performed according to the nationwide epidemiological survey manual of patients with intractable diseases (2nd edition 2006, Ministry of Health, Labour, and Welfare of Japan) as described previously [8]. PID classification was based on the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee in 2007 [2]. Patients with chronic benign neutropenia and syndrome of periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis were excluded because these were considered to be acquired diseases. The survey was conducted on PID patients who were alive on December 1, 2008 and those who were newly diagnosed and dead between December 1, 2007 and November 30, 2008 in Japan. Among the 2,291 pediatric departments and 8,026 internal medicine departments in Japan, hospitals participating in the survey were randomly selected after setting the selection ratio according to the number of beds (overall selection rate: 53.4% for pediatric departments, 20.8% for internal medicine departments; Table I). University hospitals and pediatric training hospitals, where many PID patients were considered to be treated, were stratified separately (Table I). Primary questionnaires regarding the number of patients and disease names based on PID classification were sent to the selected hospitals. Secondary questionnaires regarding age, gender, clinical manifestations, and complications of individual PID patients were sent to respondents who answered that they observed at least one PID patient with characteristics listed in the primary questionnaires.

Results

Questionnaires were distributed to 1,224 pediatric departments and 1,670 internal medicine departments of hospitals in Japan, and the response rate was 55.0% and 20.1%, respectively (Table I). A total of 1,240 patients (1,146 patients from pediatric departments and 94 patients from internal medicine departments) were registered (Table I). The estimated number of patients with PIDs in Japan was 2,900 (95% confidence interval: 2,300–3,500), and the prevalence was 2.3 per 100,000 inhabitants. We also determined the regional distribution on the basis of the patients' addresses. The estimated regional prevalence ranged from 1.7 to 4.0 per 100,000 inhabitants, and no significant differences were observed between different regions in Japan (Fig. 1). The most common form of PID was predominantly antibody deficiencies (40%), followed by congenital defects of phagocyte number, function, or both (19%) and other well-defined immunodeficiency syndromes (16%; Table II). Autoinflammatory disorders were observed in 108 cases (9%). The most common PID was Bruton's tyrosine kinase (BTK) deficiency (182 cases, 14.7%), followed by chronic granulomatous disease (CGD; 147 cases, 11.9%). However, common variable immunodeficiency disease (CVID) and selective IgA deficiency (SIgAD) were observed only in 136 (11.0%) and 49 cases (4.0%), respectively. Among patients registered from internal medicine departments, antibody deficiencies were the most common disorder (71%).

In the secondary survey, 923 cases were registered. The male-to-female ratio was 2.3:1 (n=914, unanswered: 9 cases) with a median age of 12.8 years (range: 0 to 75 years; n= 897, unanswered: 26 cases). The number of adolescent or

Table I Stratification and selection of hospitals and the survey results

	Stratification	Departments in Japan	Departments selected	Selection rate (%)	Return[a]	Response	Response rate (%)	PID Patient	Patients per department	Patients estimated
Pediatrics	University hospital	118	118	100	0	80	67.8	661	8.3	975
	Training hospital	402	402	100	4	242	60.8	376	1.6	618
	≥500 beds	92	92	100	5	48	55.2	24	0.5	44
	400–499 beds	118	118	100	3	63	54.8	42	0.7	77
	300–399 beds	287	230	80.1	4	122	54.0	31	0.3	72
	200–299 beds	289	116	40.1	4	53	47.3	6	0.1	32
	100–199 beds	486	98	20.2	0	44	44.9	4	0.1	44
	<99 beds	499	50	10.0	1	10	20.4	2	0.2	100
	Subtotal	2,291	1,224	53.4	21	662	55.0	1,146	1.7	1,961
Internal medicine	University hospital	156	156	100	1	47	30.3	37	0.8	122
	≥500 beds	374	374	100	1	86	23.1	35	0.4	152
	400–499 beds	328	263	80	1	54	20.6	6	0.1	36
	300–399 beds	692	278	40.2	6	49	18.0	10	0.2	140
	200–299 beds	1,008	202	20.0	0	36	17.8	2	0.1	56
	100–199 beds	2,460	246	10.0	1	36	14.7	1	0.0	68
	<99 beds	3,008	151	5.0	6	24	16.6	3	0.1	375
	Subtotal	8,026	1,670	20.8	16	332	20.1	94	0.3	950
Total		10,317	2,894	28.1	37	994	34.8	1,240		2,911

[a] Due to the closure of departments

adult cases (≥15 years) was 384 (42.8%; Fig. 2a). The male-to-female ratio of the younger generation (<15 years) was 2.7:1, while that of the older generation (≥15 years) was 2.0:1. Combined T and B cell immunodeficiencies (CIDs) were predominantly observed in the younger generation, while antibody deficiencies were more common with

Region	Reported PID Patients	Population ($\times 10^5$)	Estimated prevalence per 10^5 (95% C.I.)
Hokkaido	73	55.4	4.0 (0.2-7.8)
Tohoku	81	94.3	2.1 (0.9-3.3)
Kanto	387	419.8	1.8 (1.2-2.5)
Chubu	236	236.9	2.3 (1.4-3.1)
Kinki	158	208.4	2.0 (1.2-2.8)
Chugoku/Shikoku	105	116.1	1.7 (1.0-2.3)
Kyushu/Okinawa	200	146.0	2.5 (1.7-3.3)
Total	1240	1276.9	2.3 (1.8-2.7)

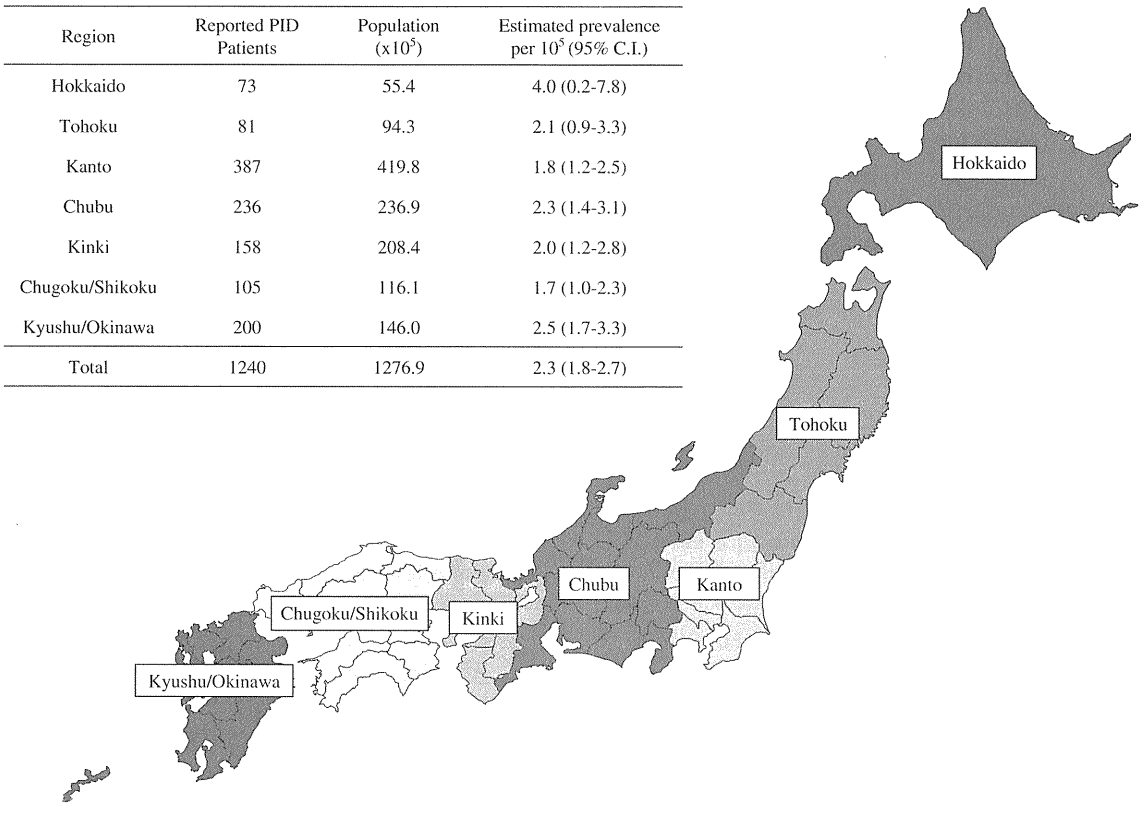

Fig. 1 Regional distribution of PID patients. *CI* Confidence interval

Table II Reported number of PID

Category	Total number	Pediatric department	Internal medicine department
I. Combined T and B cell immunodeficiencies	93 (7%)	93 (8%)	0 (0%)
γc deficiency	47	47	0
Adenosine deaminase deficiency	9	9	0
Omenn syndrome	4	4	0
Others	23	23	0
Untested or undetermined	10	10	0
II. Predominantly antibody deficiencies	501 (40%)	434 (38%)	67 (71%)
BTK deficiency	182	173	9
Common variable immunodeficiency disorders	136	107	29
Selective IgG subclass deficiency	66	58	8
Selective IgA deficiency	49	34	15
Hyper IgM syndrome	34	34	0
Transient hypogammaglobulinemia of infancy	7	7	0
Others	11	7	4
Untested or undetermined	16	14	2
III. Other well-defined immunodeficiency syndromes	194 (16%)	189 (17%)	5 (5%)
Wiskott–Aldrich syndrome	60	60	0
DNA repair defects (other than those in category I)	15	15	0
DiGeorge anomaly	38	38	0
Hyper-IgE syndrome	56	52	4
Chronic mucocutaneous candidiasis	17	16	1
Others	5	5	0
Untested or undetermined	3	3	0
IV. Diseases of immune dysregulation	49 (4%)	48 (4%)	1 (1%)
Chediak–Higashi syndrome	9	8	1
Familial hemophagocytic lymphohistiocytosis syndrome	5	5	0
X-linked lymphoproliferative syndrome	8	8	0
Autoimmune lymphoproliferative syndrome	8	8	0
APECED	4	4	0
IPEX syndrome	7	7	0
Others	2	2	0
Untested or undetermined	6	6	0
V. Congenital defects of phagocyte number, function, or both	230 (19%)	223 (19%)	7 (8%)
Severe congenital neutropenia	44	42	2
Cyclic neutropenia	19	17	2
Chronic granulomatous disease	147	144	3
Mendelian susceptibility to mycobacterial disease	5	5	0
Others	9	9	0
Untested or undetermined	6	6	0
VI. Defects in innate immunity	15 (1%)	15 (1%)	0
Anhidrotic ectodermal dysplasia with immunodeficiency	7	7	0
Interleukin-1 receptor-associated kinase 4 deficiency	2	2	0
Others	5	5	0
Untested or undetermined	1	1	0
VII. Autoinflammatory disorders	108 (9%)	101 (9%)	7 (8%)
Familial Mediterranean fever	44	40	4
TNF receptor-associated periodic syndrome	13	12	1
Hyper IgD syndrome	4	4	0
Cryopyrin-associated periodic syndrome	22	22	0

Table II (continued)

Category	Total number	Pediatric department	Internal medicine department
Others	3	3	0
Untested or undetermined	22	20	2
VIII. Complement deficiencies	32 (3%)	29 (3%)	3 (3%)
IX. Undetermined	18 (1%)	14 (1%)	4 (4%)
Total	1,240	1,146	94

APECED Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy, *IPEX* immune dysregulation, polyendocrinopathy, enteropathy, X-linked

increasing age (Fig. 2b). The median age of CID, BTK deficiency, CVID, and CGD patients was 5.2, 12.8, 25.1, and 14.7 years, respectively.

It is well known that PID patients are susceptible to many pathogens and experience community-acquired or opportunistic infections. In this study, we focused on noninfectious complications of PID because they have been less well studied on a large scale and may provide important information for improving the quality of life of PID patients. Twenty-five PID patients developed malignant disorders (2.7%; Table III). Lymphoma, in particular, Epstein–Barr virus-related, and leukemia were dominant, while there were no patients with gastric carcinoma. CVID, Wiskott–Aldrich syndrome (WAS), and ataxia telangiectasia were more frequently associated with malignant diseases among PID patients. A case of Mendelian susceptibility

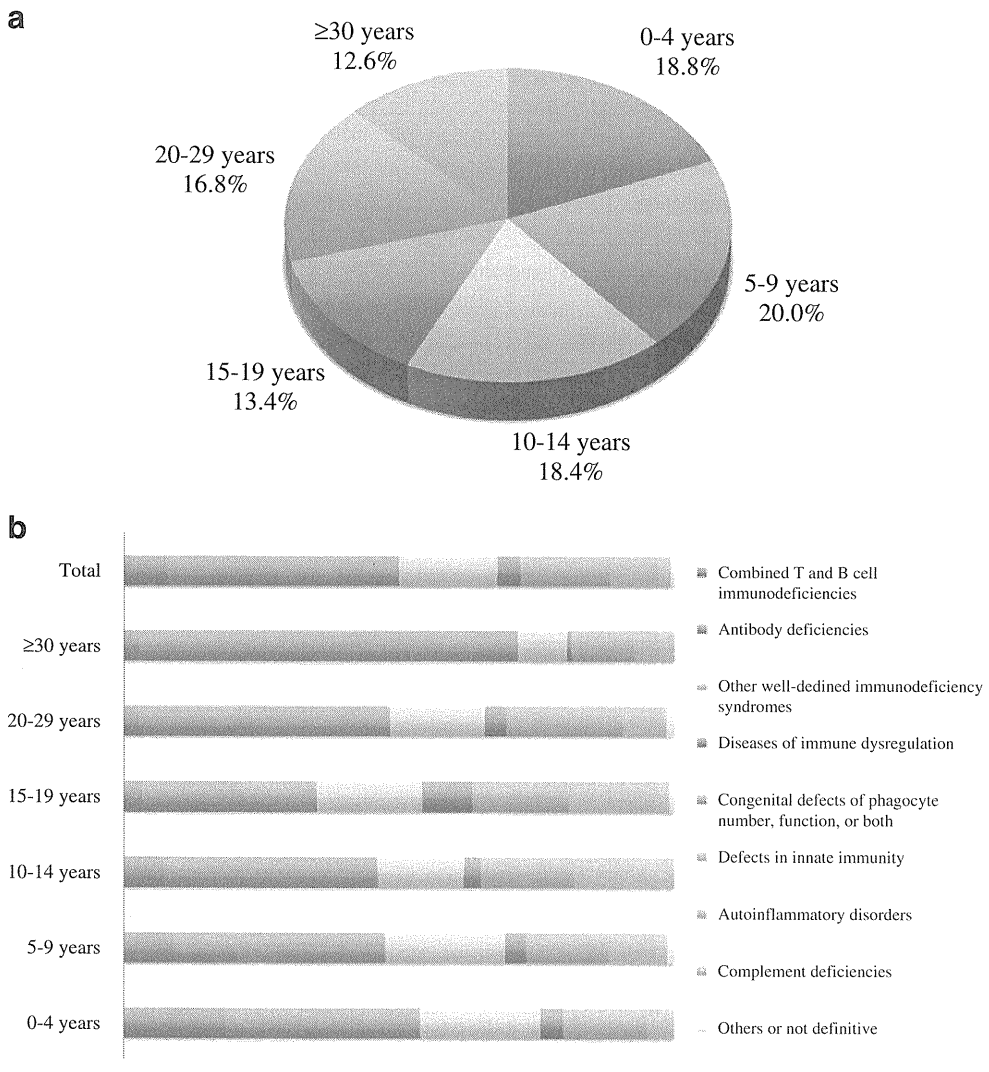

Fig. 2 **a** Age distribution of PID patients. **b** Distribution of PID in each age group

to mycobacterial disease with squamous cell carcinoma was also observed [9] (Table III).

Seventy-eight PID patients had immune-related (autoimmune) diseases (8.5%; Table IVa). Autoimmune lymphoproliferative syndrome, immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, and nuclear factor kappa B essential modulator (NEMO) deficiency were associated with immune-related diseases at a very high incidence. In addition, immune-related diseases were relatively common in CGD and CVID patients (Table IVa). The most commonly observed immune-related disease was inflammatory bowel disease (33 cases), which was most frequently observed in CGD patients, followed by immune thrombocytopenic purpura (13 cases), autoimmune hemolytic anemia (8 cases), and systemic lupus erythematosus (SLE; 8 cases; Table IVa and b). Kawasaki disease occurred in WAS and CGD patients. In addition, this is the first report of Kawasaki disease in patients with complement deficiency (C9) and familial Mediterranean fever (FMF). A patient with warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome and a patient with tumor necrosis factor receptor-associated periodic syndrome (TRAPS) were first reported as cases of type 1 diabetes mellitus and SLE, respectively [10, 11].

Discussion

We conducted a nationwide survey of PID for the first time in 30 years and report the prevalence of PID in Japan. We registered 1,240 PID patients and found that the estimated prevalence of PID (2.3/100,000) is higher than that previously reported (1.0/100,000) in Japan. Our results are equivalent to those reported in Singapore (2.7/100,000) and Taiwan (0.77–2.17/100,000) [12–14]. However, our values are lower than those reported in Middle Eastern countries such as Kuwait (11.98/100,000) or in European countries such as France (4.4/100,000) [5–7, 15]. The high rate of consanguinity may be a cause of the high prevalence rate of PID reported in Middle Eastern countries [6, 15]. There may has been sample selection bias in this study because some asymptomatic cases (SIgAD, etc.), clinically recovered cases (transient hypogammaglobulinemia of infancy, etc.), and cases in which patients were deceased were not registered. In addition, lack of recognition of PID in internal medicine departments, not just the low response rate, might also have influenced the estimated prevalence of PID as well as the age and disease distribution. The regional prevalence of PIDs in Japan was homogenous, unlike in other countries in which a higher prevalence was

Table III Malignancies in PID patients

Primary immunodeficiency	Total	*n*	Malignancy
I. Combined T and B cell immunodeficiencies	75	2	(2.7%)
Ommen syndrome	3	1	NHL (EBV+) 1[a]
Adenosine deaminase deficiency	4	1	Breast carcinoma 1
II. Predominantly antibody deficiencies	378	8	(2.1%)
Common variable immunodeficiency disorders	93	7	HL 2, ML 2, ALL 1, Basal cell carcinoma 1, Cervical carcinoma 1
Good syndrome	4	1	Double primary carcinoma of breast and colon 1
III. Other well-defined immunodeficiency syndromes	165	7	(4.2%)
Wiskott–Aldrich syndrome	57	5	NHL 3, NHL/HL 1, LPD (EBV-) 1
Ataxia telangiectasia	13	2	T-ALL 1, MDS 1
IV. Diseases of immune dysregulation	38	4	(10.5%)
X-linked lymphoproliferative syndrome	5	2	Burkitt lymphoma 2
Autoimmune lymphoproliferative syndrome	6	2	HL (EBV+) 1, Brain tumor 1
V. Congenital defects of phagocyte number, function, or both	153	4	(2.6%)
Severe congenital neutropenia	35	3	MDS 3 (including 2 cases with monosomy 7)
MSMD	7	1	Squamous cell carcinoma of finger 1
VI. Defects in innate immunity	12	0	(0%)
VII. Autoinflammatory disorders	74	0	(0%)
VIII. Complement deficiencies	23	0	(0%)
IX. Undetermined	5	0	(0%)
Total	923	25	(2.7%)

n Number of PID patients who had malignant disorders, *ALL* acute lymphoblastic leukemia, *EBV* Epstein-Barr virus, *HL* Hodgkin lymphoma, *LPD* lymphoproliferative disease, *MDS* myelodysplastic syndrome, *ML* malignant lymphoma, *MSMD* Mendelian susceptibility to mycobacterial disease, *NHL* non-Hodgkin lymphoma

[a] The number of patients

Table IV Immune-related diseases in PID patients

(a) Immune-related diseases with each PID

Primary immunodeficiency	Total	*n*	Immune-related disease
I. Combined T and B cell immunodeficiencies	75	2	(2.6%)
MHC class II deficiency (suspected)	1	1	ITP with AIHA 1[a]
CD4 deficiency	1	1	Hashimoto disease 1
II. Predominantly antibody deficiencies	378	24	(6.3%)
Common variable immunodeficiency disorders	93	16	ITP 3, RA 2, AIHA 2, Hashimoto's disease 2, IBD 2, SLE 1, MG 1, ADEM 1, Autoimmune hepatitis 1, Uveitis 1
Hyper-IgM syndrome	32	3	JIA 1, SLE (complicated with C1q deficiency) 1, IBD 1
Selective IgA deficiency	28	3	SLE 1, SLE with Kikuchi disease 1, RA 1
IgG subclass deficiency	50	2	ITP with AIHA 1, ITP with MS 1
III. Other well-defined immunodeficiency syndromes	165	5	(3.0%)
Wiskott–Aldrich syndrome	57	3	AIHA 2, Kawasaki disease 1
DiGeorge syndrome	33	2	AIHA 1, ITP 1
IV. Diseases of immune dysregulation	38	10	(26.3%)
X-linked lymphoproliferative syndrome	5	1	IBD 1
Autoimmune lymphoproliferative syndrome	6	4	ITP 3, Graves' disease with IBD 1
APECED	5	1	T1DM with Hashimoto's disease and Vogt–Koyanagi–Harada disease 1
IPEX syndrome	6	4	T1DM 1, T1DM with ITP, AIN and IBD 1, Autoimmune enteritis 1, AIHA with Autoimmune enteritis and Hashimoto's disease 1
V. Congenital defects of phagocyte number, function, or both	153	25	(16.3%)
Chronic granulomatous disease	87	25	IBD 20, ITP 2, JIA 1, MCTD 1, Kawasaki disease 1
VI. Defects in innate immunity	12	5	(41.7%)
NEMO deficiency	7	4	IBD 3, IBD with JIA 1
WHIM syndrome	3	1	T1DM 1
VII. Autoinflammatory disorders	74	3	(4.0%)
Familial Mediterranean fever	36	2	SLE 1, Kawasaki disease 1
TNF receptor associated periodic syndrome	9	1	SLE 1
VIII. Complement deficiencies	23	3	(13.0%)
C4 deficiency	1	1	SLE with RA 1
C6 deficiency	1	1	IBD 1
C9 deficiency	11	1	Kawasaki disease 1
IX. Undetermined	5	1	(20%)
Nakajo syndrome	1	1	SLE 1
Total	923	78	(8.5 %)

(b) Immune-related manifestations associated with PID

Immune-related diseases	*n*
IBD (including autoimmune enteritis)	33
ITP	13
AIHA	8
SLE	8
RA/JIA	6
Hashimoto's disease/Graves' disease	5
Kawasaki disease	4
T1DM	4
Uveitis (including Vogt–Koyanagi–Harada disease)	2
ADEM/MS	2
Others	5

n Number of PID patients who had immune-related disorders, *ADEM* acute disseminated encephalomyelitis, *AIHA* autoimmune hemolytic anemia, *AIN* autoimmune neutropenia, *APECED* autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, *IBD* inflammatory bowel disease, *IPEX* immunodysregulation, polyendocrinopathy, enteropathy X-linked, *ITP* immune thrombocytopenic purpura, *JIA* juvenile idiopathic arthritis, *MCTD* mixed connective tissue disease, *MG* myasthenia gravis, *MS* multiple sclerosis, *RA* rheumatoid arthritis, *SLE* systemic lupus erythematosus, *T1DM* type 1 diabetes mellitus, *WHIM* warts, hypogammaglobulinemia, infections, and myelokathexis

[a] The number of patients

observed in urban areas [5, 7, 16]. This may be because many PID patients were treated or followed by PID specialists distributed nationwide in Japan; this is assumed by the location of hospitals with which they were affiliated.

The distribution ratios of BTK deficiency (14.7%) and CGD (11.9%) in Japan were higher than those in a previous report from Europe (5.87% and 4.33%, respectively), while those of CIDs and other well-defined immunodeficiency syndromes were comparable [17]. The prevalence of BTK deficiency was previously reported to be 1/900,000–1,400,000 in a European cohort study [18]. In contrast, this value was estimated to be 1/300,000 in Japan in our study. BTK deficiency appears to be common in Japan, although this may be partially because more patients, including those showing atypical clinical manifestations, were diagnosed more accurately by the recently established genetic diagnostic network in Japan [19]. This is supported by the highest proportion of Japanese patients in the international mutation database for X-linked agammaglobulinemia (BTKbase) [20]. The reason for the low number of registered CGD patients in Europe in a recent report (1/620,000) [17] is unknown; the prevalence of CGD was 1 in 250,000 in a previous European survey [21], which was similar to our results (1 in 380,000 in this study and 1 in 280,000 in our previous study [22]). The percentage of BTK deficiency and CGD would be lower if more adult cases were registered because the prevalence of these disorders is low in adults. CVID was the most commonly reported PID (20.7%) in Europe, and the onset of symptoms was observed most commonly in the third decade of life in these patients [17, 23]. In this study, CVID constituted 11.0% (136 cases) of PID cases, and only 29 cases were reported from internal medicine departments (Table II). A lower number of registered CVID patients may have led to a lower number of reported patients with antibody deficiency and a lower prevalence of PID, although it is still possible that CVID is not as common in Japan as in European countries. There was no significant difference in the distribution rate of SIgAD between Japanese and Europeans, although SIgAD is rare in Japanese (1/18,500) compared with Caucasians (1/330–2,200) according to seroepidemiologic studies [24]. This may be because most SIgAD patients lack clinical manifestations. The distribution ratio of autoinflammatory disorders in Japan (9%) was much higher than that in Europe (1.02%) [17] (Table II). Considering the disease type of the autoinflammatory disorders was not specified in 22 cases (20%), it is possible that many other patients with autoinflammatory disorders remain undiagnosed in Japan as well as in other countries.

The percentage of men (69.7%) with PID is higher in Japan than in Europe (60.8%) or Kuwait (61.8%), but is equivalent to that in Taiwan (70.2%) [6, 13, 17]. The higher ratio of men, particularly in younger generation (<15 years), appears to be due to the larger number of X-linked PID patients (BTK deficiency, X-CGD, γc deficiency, etc.) in this study compared to that in Europe or Kuwait. Adolescents or adults (≥15 years) constituted 42.8% of the patients in this study, which is equivalent to the number in the European study (≥16 years: 46.6%), while those >16 years constituted only 10.9% in the previous survey [3, 17]. In this study, it was found that CVID and SIgAD are common in adults (Table II) and that antibody deficiencies are more common with increasing age (Fig. 2b). A reason for the increased number of adult PID patients may be long-term survival of PID patients due to improved treatments such as immunoglobulin replacement therapy. In addition, an increased likelihood of patients being diagnosed by internists as having late-onset PID, e.g., CVID and SIgAD, may have contributed to these values [17, 25, 26]. Therefore, it is important for internists to be well-informed regarding PID. In contrast, CIDs are fatal during infancy without hematopoietic stem cell transplantation or gene therapy. Because hematopoietic stem cell transplantation has been widely performed in Japan since the 1990s, surviving patients with CID are limited to the younger generation, similar to French patients (Fig. 2b) [5, 27, 28].

It has been reported that PID patients are at increased risk of developing malignant diseases, in particular, non-Hodgkin lymphoma, leukemia, and stomach cancer [29]. Although lymphoma and leukemia were relatively common, stomach cancer was not observed in our study. In the previous survey in Japan, eight of nine PID patients with malignant disorders (including one gastric cancer patient) died [3]. It is possible that some PID patients with malignant disorders were not registered because they were deceased. PID is also associated with immune-related diseases because of a defect in the mechanisms to control self-reactive B and T cells. The frequency of immune-related manifestations varied among individual PID patients, as reported previously [30, 31]. Four PID patients who had developed Kawasaki disease, one patient with WHIM syndrome and type 1 diabetes mellitus, and one patient with TRAPS and SLE in our study may provide new pathophysiological insights of these diseases and the association between PID and autoimmune diseases.

Conclusions

We report the prevalence and clinical characteristics of PIDs in Japan. Although the advances in diagnostic technologies and treatments have improved the prognoses of PID, many patients continue to experience severe complications such as malignancy and immune-related diseases as well as infections. To improve the quality of life of PID patients, it is necessary to pay attention to

complications and treat them appropriately. Web-based PID databases and consultation systems have been created in Japan (Primary Immunodeficiency Database in Japan [4] and Resource of Asian Primary Immunodeficiency Diseases in Asian countries [32]) to reveal precise information regarding PID and to promote cooperation between doctors and researchers [19].

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Conflict of Interest There is no actual or potential conflict of interest in relation to the study.

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In conclusion, the associations among asthma, biofilm-forming bacteria, and revision ESS are strong and robust after adjusting for other factors in patients with CRS from a tertiary medical center. Despite its limitations, this study may improve our understanding of refractory CRS pathogenesis, possibly leading to more effective treatment strategies, such as incorporating the treatments of asthma and biofilm infection into conventional CRS therapies. Prospective cohort studies in diverse populations are needed to assess the causality of these associations.

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Quantification of κ-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects

To the Editor:

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency caused by severely decreased numbers of mature peripheral B lymphocytes as a result of a mutation in the *BTK* gene. Non-XLA is characterized by hypogammaglobulinemia with decreased B-cell counts (less than 2% of mature B cells) in the absence of the *BTK* gene mutation. Both XLA and non-XLA are caused by an early B-cell maturation defect.[1] In patients with XLA and non-XLA, recurrent infections appear between 3 and 18 months of age, whereas the mean age at diagnosis is 3 years.[2] This delayed diagnosis results in frequent hospitalization because of pneumonia, sepsis, meningitis, and other bacterial infections, which frequently require intravenous administration of antibiotics and can be fatal. Frequent pneumonia results in a high incidence of chronic lung diseases.[3] Thus, early diagnosis and early treatment, including periodical intravenous immunoglobulin replacement therapy, is essential to improve the prognosis and the quality of life of patients with XLA and non-XLA.

In the process of B-cell maturation, immunoglobulin κ-deleting recombination excision circles (KRECs) are produced during κ-deleting recombination allelic exclusion and isotypic exclusion of the λ chain.[4] Coding joint (cj) KRECs reside within the chromosome, whereas signal joint (sj) KRECs are excised from genomic DNA. cjKREC levels remain the same after B-cell division, whereas sjKREC levels decrease, because sjKRECs are not replicated during cell division.[5] Because the B-cell maturation defects in XLA and non-XLA occur before κ-deleting recombination, KRECs are not supposed to be produced. Therefore, measurements of KRECs have the potential to be applied to the identification of these types of B-cell deficiencies in patients, which consist of around 20% of all B-cell defects.[6] In addition, some types of combined immunodeficiencies show an arrest in B-cell maturation and can also be identified by this method. The success of newborn screening for T-cell deficiencies by measuring T-cell–receptor excision circles[7] prompted us to develop a newborn screening method for XLA and non-XLA by measuring KRECs derived from neonatal Guthrie cards.

The study protocol was approved by the National Defense Medical College institutional review board, and written informed consent was obtained from the parents of normal controls, the affected children, and adult patients, in accordance with the Declaration of Helsinki.

First, we determined the sensitivity of detection levels of cjKRECs and sjKRECs in Guthrie cards using real-time quantitative PCR.[5] Normal B cells from a healthy adult were isolated from peripheral blood (PB; mean purity, 88.5%). PB was also obtained from 1 patient with XLA (P20) whose B-cell number was 0.09 in 1 μL whole blood and who was negative for sjKRECs ($<1.0 \times 10^2$ copies/μg DNA). Various numbers of normal B cells were serially added to 1 mL whole PB obtained from this patient with XLA. The B-cell–added XLA whole blood was then applied to filter papers, and 3 punches (3 mm in diameter) of dried blood spots were used for DNA extraction. At least 3 DNA samples containing the same B-cell concentrations (0.09-400 B cells/μL) were used for the real-time quantitative PCR of cjKRECs and sjKRECs. The percentages of the positive samples ($>1.0 \times 10^2$ copies/μg DNA) of cjKRECs and sjKRECs increased constantly