most cases of HIES are caused by dominant-negative (DN) mutations of the *STAT3* gene (Holland et al., 2007; Minegishi et al., 2007). However, the pathogenesis of this syndrome remains unclear. In particular, the molecular mechanisms underlying the allergic manifestations, including atopic dermatitis and extremely high serum IgE levels, remain one of the great enigmas in the pathogenesis of this syndrome.

STAT3 is a transcription factor that binds to the promoter regions of various genes, including those encoding acute-phase proteins. STAT3 plays a critical role in signal transduction for many cytokines, including those of the γc family (IL-2, IL-7, IL-9, IL-15, and IL-21), the gp130 family (IL-6, IL-11, IL-27, and IL-31), the IL-10 family (IL-10 and IL-22), and receptor-type tyrosine kinases. The systemic deletion of STAT3 in mice is lethal, but studies involving the tissue-specific deletion of STAT3 have demonstrated that STAT3 plays a critical role in cell migration, survival, proliferation, apoptosis, inflammation, and tumorigenesis in many tissues (Akira, 2000). Furthermore, recent data unanimously demonstrated that STAT3 plays an essential role for Th17 cell development in humans (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008; Minegishi et al., 2009), which could explain, at least in part, why HIES patients suffer from recurrent staphylococcal infections confined to the skin and lung (Minegishi et al., 2009).

Allergic diseases may result from an inappropriate balance between effector Th2 cells and T_{reg} cells (Umetsu and DeKruyff, 2006; Akdis and Akdis, 2009; Lloyd and Hawrylowicz, 2009). Th2 cells respond to allergens and produce IL-4, IL-5, IL-9, and IL-13. Th2 cytokines induce changes in blood vessels that lead to the up-regulation of intercellular adhesion molecule 1 and vascular cell-adhesion molecule 1, in turn leading to the recruitment of very late antigen 4–expressing eosinophils. These factors also induce the survival and activation of eosinophils. In addition, IL-4 and IL-13 are responsible for promoting Ig class switching to IgE (Hammad and Lambrecht, 2008). Newly identified cytokines such as IL-25, IL-31, and IL-33 also participate in Th2 cell–mediated inflammation (Dillon et al., 2004; Wang et al., 2007; Kakkar and Lee, 2008). Th1 cells may also contribute to allergic inflammation by inducing the apoptosis of epithelial cells in atopic dermatitis (Trautmann et al., 2000).

T_{reg} cells are key mediators of peripheral tolerance that actively suppress effector T cells and inhibit immune response–mediated tissue damage. Both $FOXP3^+$ T_{reg} cells and IL-10–producing $FOXP3^-$ T_{reg} cells play an essential role in the regulation of allergic inflammation (Curotto de Lafaille et al., 2001; Zheng and Rudensky, 2007; Sakaguchi et al., 2008). There are two types of $FOXP3^+$ T_{reg} cells: natural T_{reg} cells (nT_{reg} cells) and induced T_{reg} cells (iT_{reg} cells). nT_{reg} cells develop in the thymus, whereas iT_{reg} cells develop in the periphery. In the presence of TGF-β1, naive $FOXP3^-$ $CD4^+$ T cells are converted into $FOXP3^+$ iT_{reg} cells (Chen et al., 2003; Coombes et al., 2007; Rubtsov and Rudensky, 2007; Zheng et al., 2007). Mutations in the human *FOXP3* gene result in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Bennett et al., 2001; Wildin et al., 2001). Patients with IPEX syndrome suffer from enteropathy, autoimmune diabetes and thyroiditis, food allergy, and atopic dermatitis with extremely high serum IgE levels. FOXP3 deficiency in mice also leads to atopic manifestations (Fontenot et al., 2003; Lin et al., 2005).

DCs are central to the orchestration of the various types of immunity and tolerance (Banchereau et al., 2000; Kapsenberg, 2003; Steinman et al., 2003). Immature DCs function as sentinels in the periphery, undergoing terminal differentiation in response to various danger signals. Maturing DCs migrate to the lymph nodes, where they acquire potent antigen-presenting capacity and induce vigorous T cell responses by expressing co-stimulatory molecules and secreting large amounts of proinflammatory cytokines. The interaction between DCs and naive $CD4^+$ T cells is considered to determine the fate of $CD4^+$ T cells. Cytokines produced by DCs, such as IL-12 and IFN-α, may bias $CD4^+$ T cell priming toward the Th1 pathway (Schulz et al., 2000). Notch ligands, such as Jagged 1, expressed by DCs may promote $CD4^+$ T cells toward the Th2 pathway (Amsen et al., 2009). In addition, DCs play a key role in the induction and maintenance of peripheral T cell tolerance (Steinman et al., 2003; Rutella et al., 2006).

We investigated the molecular mechanism underlying the atopic manifestations in HIES by studying Th1–Th2–T_{reg} cell balance and the development and function of primary and monocyte-derived DCs (MoDCs). The results suggest that IL-10 signaling by DCs may be crucial for the generation of tolerogenic DCs and iT_{reg} cells for the maintenance of an appropriate Th1–Th2–T_{reg} cell balance in vivo in humans.

RESULTS

Normal Th1 and Th2 differentiation from naive $CD4^+$ T cells but increased Th2 cytokine production from activated T cells in PBMCs of *STAT3* patients

We first evaluated Th1 and Th2 cell development of naive $CD4^+$ T cells in *STAT3* patients. Naive $CD4^+$ T cells were unstimulated or stimulated with anti-CD3 and anti-CD28 (anti-CD3/CD28) mAbs under neutral, Th1, and Th2 differentiation conditions, and the development of IFN-γ– and IL-4–producing cells was evaluated by cytoplasmic staining and flow cytometry. The development of Th1 and Th2 cells was similar in control subjects and *STAT3* patients (Fig. S1 A). This observation was confirmed by ELISA of the culture supernatants of naive $CD4^+$ T cells, showing similar levels of IFN-γ, IL-5, and IL-13 secretion for control subjects and *STAT3* patients (Fig. S1 B). We next evaluated Th1 and Th2 cytokine production from PBMCs after stimulation with anti-CD3/CD28 mAbs. The production of IFN-γ was equivalent between control subjects and *STAT3* patients, but the production of IL-5 and IL-13 was increased in *STAT3* patients compared with control subjects (Fig. S1 C). These results suggest that cells in PBMCs other than naive $CD4^+$ T cells are likely to be responsible for increased Th2 cytokine production in *STAT3* patients.

The number and suppressive activity of T_{reg} cells in the peripheral blood are normal in *STAT3* patients

We next evaluated the number of FOXP3$^+$ T_{reg} cells among PBMCs because STAT3 is involved in the transduction of IL-6 and IL-21 signals, which may influence the balance between nT_{reg} cell and Th17 cell differentiation (Harrington et al., 2005; Bettelli et al., 2006; Ivanov et al., 2006; Veldhoen et al., 2006). Similar numbers of PBMCs were obtained from control subjects and *STAT3* patients, and these cells were stained for extracellular CD4 and CD25 and intracellular FOXP3 and evaluated by flow cytometry. The percentages of CD4$^+$CD25$^+$ cells and CD4$^+$FOXP3$^+$ cells did not differ significantly between control subjects and *STAT3* patients (Fig. 1 A).

We then investigated the function of T_{reg} cells in the peripheral blood ex vivo. CD4$^+$CD25$^+$CD62L$^+$ T_{reg} cells were obtained from the peripheral blood of control subjects and *STAT3* patients at a purity of >99% and were co-cultured with autologous CD4$^+$CD25$^-$CD62L$^+$ responder T cells in the presence or absence of anti-CD3/CD28 mAbs. The addition of 1.25×10^3 control T_{reg} cells to the 1.25×10^4 control responder T cells resulted in levels of [^{3}H]thymidine incorporation 55% lower than those obtained after the addition of 1.25×10^3 control responder T cells. Levels of [^{3}H]thymidine incorporation were similarly lowered by the addition of 1.25×10^3 patient T_{reg} cells to the 1.25×10^4 patient responder T cells (Fig. 1 B). Modification of the ratio of T_{reg} cells to responder T cells from 1:1 (T_{reg} cell/responder T cell) to 1:100 resulted in no significant difference in the percent suppression of [^{3}H]thymidine incorporation between control subjects and *STAT3* patients (Fig. 1 C). These results indicate that the in vivo generation and ex vivo function of T_{reg} cells were normal in *STAT3* patients.

Defective IL-10 signaling in MoDCs from *STAT3* patients

We next evaluated the generation of MoDCs in vitro. Isolated CD14$^+$ monocytes from the PBMCs of control subjects and *STAT3* patients were cultured with GM-CSF and IL-4 for 5 d and then allowed to mature in the presence of LPS for 2 d. MoDC differentiation was normal, as shown by evaluations of the forward and side light scatter of the cells (Fig. S2 A), the expression levels of CD1a (Fig. S2 B), CD80, CD83, and CD86 (Fig. S2 C), and FITC-dextran uptake (Fig. S2 D). Of note, levels of CD86 expression before LPS stimulation were slightly higher in *STAT3* patients than in control subjects (Fig. S2 C), suggesting that autocrine IL-10 may regulate the expression of DC maturation markers in control subjects but not in *STAT3* patients (Corinti et al., 2001).

We have previously demonstrated that *STAT3* plays an important role in IL-10 signal transduction in human monocyte-derived macrophages (Minegishi et al., 2007). We therefore investigated IL-10 signal transduction in MoDCs. Consistent with our previous findings, the transcriptional

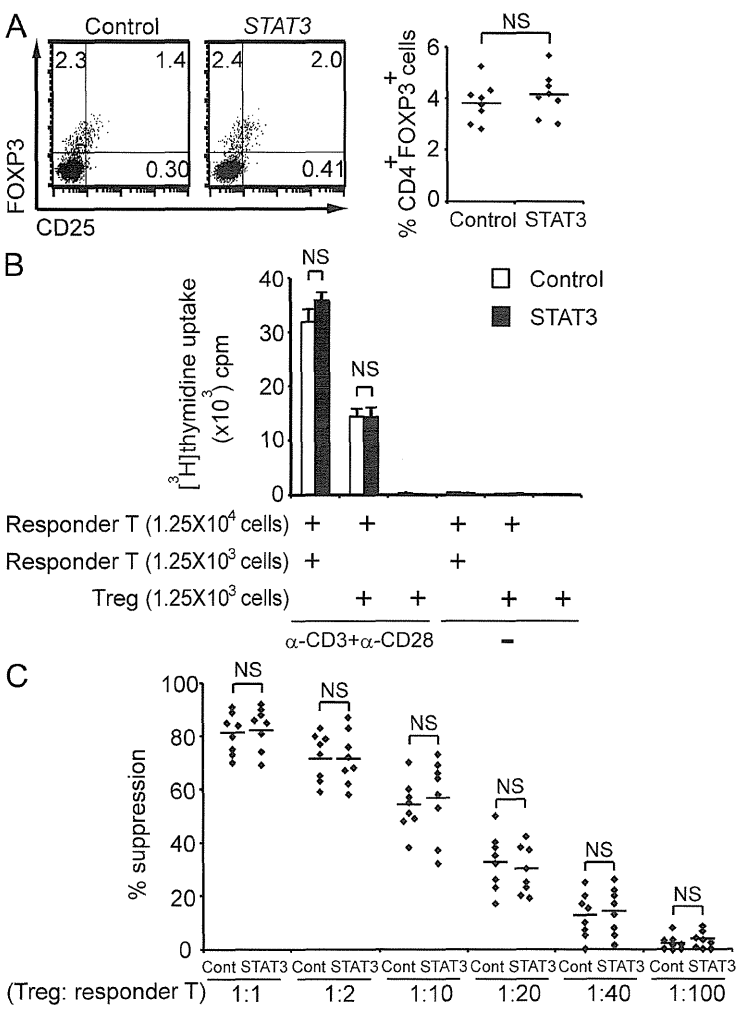

Figure 1. The number and suppressive activity of T_{reg} cells in the peripheral blood are normal in *STAT3* patients. (A, left) Representative dot plots gated on CD4$^+$ peripheral blood T cells from a control subject and a *STAT3* patient. (right) Summary data from eight control subjects and eight *STAT3* patients showing percentages of CD4$^+$FOXP3$^+$ cells. Data are representative of at least two independent experiments. (B) CD4$^+$CD25$^-$CD62L$^+$ responder T cells and CD4$^+$CD25$^+$CD62L$^+$ T_{reg} cells were isolated by cell sorting. Responder T cells and T_{reg} cells were co-cultured as indicated for 5 d with or without a 1:100 (vol/vol) dilution of anti-CD3 + anti-CD28 mAb-coated beads. For the evaluation of proliferation, 1 μCi (37 kBq) [^{3}H]thymidine was added to the culture medium for the last 18 h. Graph shows mean ± SD. (C) CD4$^+$CD25$^-$CD62L$^+$ responder T cells and CD4$^+$CD25$^+$CD62L$^+$ T_{reg} cells were isolated and cultured as in B at the indicated ratio. Summary data ($n = 8$ each) are shown. Data are representative of at least two independent experiments performed in triplicate. (A and C) Horizontal bars indicate mean values.

up-regulation of *SOCS3* and *VCAN* (*CSPG2*), two genes responsive to IL-10, was impaired in MoDCs from *STAT3* patients, as demonstrated by comparison with control subjects (Fig. 2 A). Intact signal transduction was observed for TGF-β1, the other critical inhibitory cytokine, in the MoDCs of *STAT3* patients (Fig. S2 E). We evaluated the effect of prior treatment with IL-10 on the phenotypic maturation of MoDCs. IL-10 was added to the culture medium on day 3 of DC differentiation. The prior treatment with IL-10 did not block the differentiation of MoDCs in response to GM-CSF and IL-4 (Fig. S3, A and B) but inhibited the LPS-induced maturation of MoDCs, with inhibition of the up-regulation of co-stimulatory molecules CD80 and CD86 and defective up-regulation of the DC maturation marker CD83 in a control subject. In contrast, the maturation of MoDCs derived from *STAT3* patients showed little sign of inhibition by prior treatment with IL-10 (Fig. S3 C). Up-regulation of CD80, CD83, and CD86 expression by LPS was inhibited by IL-10 pretreatment in control subjects, but the IL-10 pretreatment failed to inhibit the up-regulation of CD80, CD83, and CD86 by LPS in *STAT3* patients (Fig. 2 B). Consistent with this observation, the production of inflammatory cytokines, including TNF, IL-6, and IL-12p40, was suppressed by prior treatment with IL-10 in control subjects. The suppression by IL-10 was severely impaired in the MoDCs from *STAT3* patients (Fig. 2 C). Untreated and IL-10–treated MoDCs were harvested, extensively washed, and co-cultured with third-party allogeneic naive $CD4^+$ T cells from control subjects. LPS-stimulated mature MoDCs induced a significant increase in the uptake of [^{3}H]thymidine by naive $CD4^+$ T cells, with IL-10–treated DCs (IL-10–DCs) from a control subject markedly inhibiting the incorporation of [^{3}H]thymidine. In contrast, the down-regulation was very modest in the IL-10–treated MoDCs from *STAT3* patients. In the absence of MoDCs or naive $CD4^+$ T cells, almost no incorporation of [^{3}H]thymidine was detected (Fig. S4 A). Production of IFN-γ, IL-5, and IL-13 followed a very similar pattern, with prior IL-10 treatment inducing significant down-regulation in control subjects and barely detectable down-regulation in *STAT3* patients (Fig. 2 D). Thus, IL-10 was defective in MoDCs from *STAT3* patients, impairing suppression of (a) the up-regulation of co-stimulatory molecules on MoDCs, (b) the up-regulation of cytokine production by MoDCs, (c) the proliferation of co-cultured naive $CD4^+$ T cells, and (d) cytokine production by co-cultured naive $CD4^+$ T cells.

IL-10 signaling defect in MoDCs leads to the defective generation of tolerogenic DCs and iT_{reg} cells

Control MoDCs up-regulated the expression of inhibitory molecules, including PD-L1, PD-L2, ILT-3, and ILT-4 but not ICOS-L by IL-10 treatment. The up-regulation of these inhibitory molecules was severely impaired in the MoDCs of *STAT3* patients (Fig. 3 A). We then investigated the functional consequences of the defective up-regulation of inhibitory molecules for MoDCs by co-culturing untreated and IL-10–DCs with third-party allogeneic naive $CD4^+$ T cells from control subjects. *FOXP3* messenger RNA (mRNA) levels in $CD4^+$ T cells co-cultured with control IL-10–DCs were approximately four times higher than those for cells co-cultured with untreated control MoDCs. However, up-regulation was severely impaired when naive $CD4^+$ T cells were co-cultured with IL-10–DCs from *STAT3* patients (Fig. 3 B). This observation was confirmed by the cytoplasmic staining of FOXP3 protein and flow cytometric analysis of the $CD4^+$ T cells (Fig. 3 C). This up-regulation of FOXP3 was not likely to be mediated by simple T cell activation because naive $CD4^+$ T cells cultured with control IL-10–DCs proliferated less vigorously compared with those with control DCs and because naive $CD4^+$ T cells cultured with patient DCs proliferated more vigorously compared with those with control DCs in the absence or presence of pretreatment with IL-10 (Fig. S4 B). iT_{reg} cells from control subjects and *STAT3* patients expressed equivalent amount of CD25 on their surface, but the expression levels of CTLA-4 and GITR (glucocorticoid-induced TNFR-related) were up-regulated by the co-culture with control IL-10–DCs, but the up-regulation was impaired by the co-culture with patient IL-10–DCs (Fig. S5).

We further evaluated the consequences of defective FOXP3 up-regulation by investigating iT_{reg} cell activity. Purified $CD4^+CD25^+$ T cells from the co-culture were added to autologous $CD4^+CD25^-$ responder T cells, and the mixture was stimulated with anti-CD3/CD28 mAbs. $CD4^+CD25^+$ T cells cultured with control IL-10–DCs efficiently suppressed the proliferation of CFSE-labeled autologous responder T cells (Fig. 3 D, left, second panel). The suppression of proliferation was severely impaired by $CD4^+CD25^+$ T cells cultured with patient IL-10–DCs (Fig. 3 D, left, third panel). We further evaluated cytokine production by a co-culture of responder T cells and $CD4^+CD25^+$ T cells. The production of IFN-γ, IL-5, and IL-13 was suppressed by co-culture with $CD4^+CD25^+$ cells cultured with control IL-10–DCs (Fig. 3 E). The cytokine production was rather increased by the addition of $CD4^+CD25^+$ T cells cultured with patient IL-10–DCs, which might be caused by decreased iT_{reg} cells and increased activated T cells in this $CD4^+CD25^+$ T cell population from *STAT3* patients. Thus, the IL-10 signaling defect in MoDCs results in the impaired generation of tolerogenic DCs and iT_{reg} cells.

The generation of $FOXP3^+$ iT_{reg} cells is dependent on TGF-β1 (Chen et al., 2003; Coombes et al., 2007; Rubtsov and Rudensky, 2007; Zheng et al., 2007). We therefore investigated the relationship between IL-10–DCs and TGF-β1 in the generation of $FOXP3^+$ iT_{reg} cells. TGF-β1 in the culture medium efficiently up-regulated FOXP3 expression in naive $CD4^+$ T cells in the presence of untreated immature DCs (Fig. 3 F). Control IL-10–DCs up-regulated FOXP3 expression, equivalent to TGF-β1 (Fig. 3 F, fifth dataset vs. third dataset), and a combination of control IL-10–DCs and TGF-β1 (Fig. 3 F, seventh dataset) further up-regulated FOXP3 expression. TGF-β1 effectively up-regulated FOXP3 expression in naive $CD4^+$ T cells when co-cultured with patient DCs, but patient IL-10–DCs were inefficient for the up-regulation

of FOXP3 expression (Fig. 3 F, sixth dataset). Thus, in addition to TGF-β1, IL-10–DCs play a crucial role in the generation of FOXP3$^+$ iT$_{reg}$ cells. Moreover, these results demonstrated that the defect in FOXP3 up-regulation was not caused by the lack of TGF-β1 in IL-10–DCs from *STAT3* patients because the addition of exogenous TGF-β1 did not rescue this

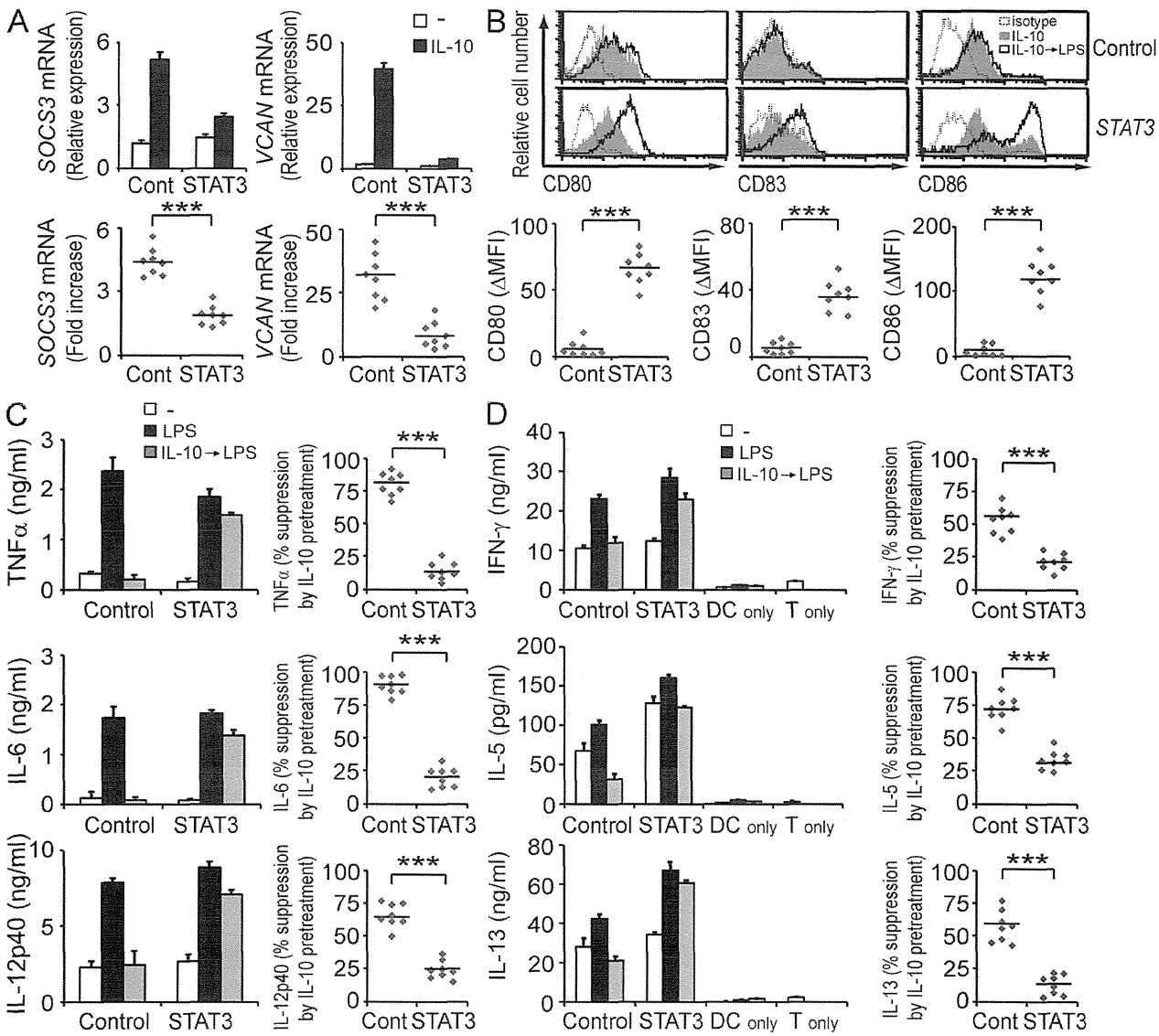

Figure 2. Defective IL-10 signaling in MoDCs from *STAT3* patients. (A) Immature DCs from a control subject and a *STAT3* patient were stimulated with or without IL-10 for 2 h, and the amounts of *SOCS3* and *VCAN* (*CSPG2*) mRNAs were analyzed by Q-PCR. The representative data are shown at the top, 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. (B) IL-10–treated MoDCs (IL-10–DCs) were generated from CD14$^+$ monocytes by culturing with GM-CSF and IL-4 in the presence of IL-10 from day 3 of the culture. Representative histograms of CD80, CD83, and CD86 expression of IL-10–DCs (IL-10) and LPS-matured MoDCs after prior treatment with IL-10 (IL-10 → LPS) from a control subject and a *STAT3* patient are at the top. Dashed lines indicate staining with isotype-matched control mAbs. Summary data ($n = 8$ each) showing delta values of mean fluorescence intensity (ΔMFI), LPS-matured IL-10–DCs minus IL-10–DCs, of CD80, CD83, and CD86 are at the bottom. (C) Representative cytokine levels in the culture supernatants of unstimulated immature MoDCs, LPS-matured MoDCs (LPS), and LPS-matured MoDCs after prior treatment with IL-10 (IL-10 → LPS) from a control subject and a *STAT3* patient are on the left. Summary data ($n = 8$ each) showing percent suppression are on the right. All samples were evaluated in triplicate. (D) Third-party allogeneic naive CD4$^+$ T cells from control subjects were co-cultured with immature DCs (−), LPS-matured DCs (LPS), or LPS-matured DCs after prior treatment with IL-10 (IL-10 → LPS), and the levels of the cytokines were evaluated by ELISA in triplicate. Representative data are on the left, and summary data of percent suppression by IL-10 pretreatment ($n = 8$ each) are on the right. Data are representative of at least two independent experiments. (A, C, and D) Graphs show mean ± SD. (A–D) Horizontal bars indicate mean values. ***, $P < 0.001$.

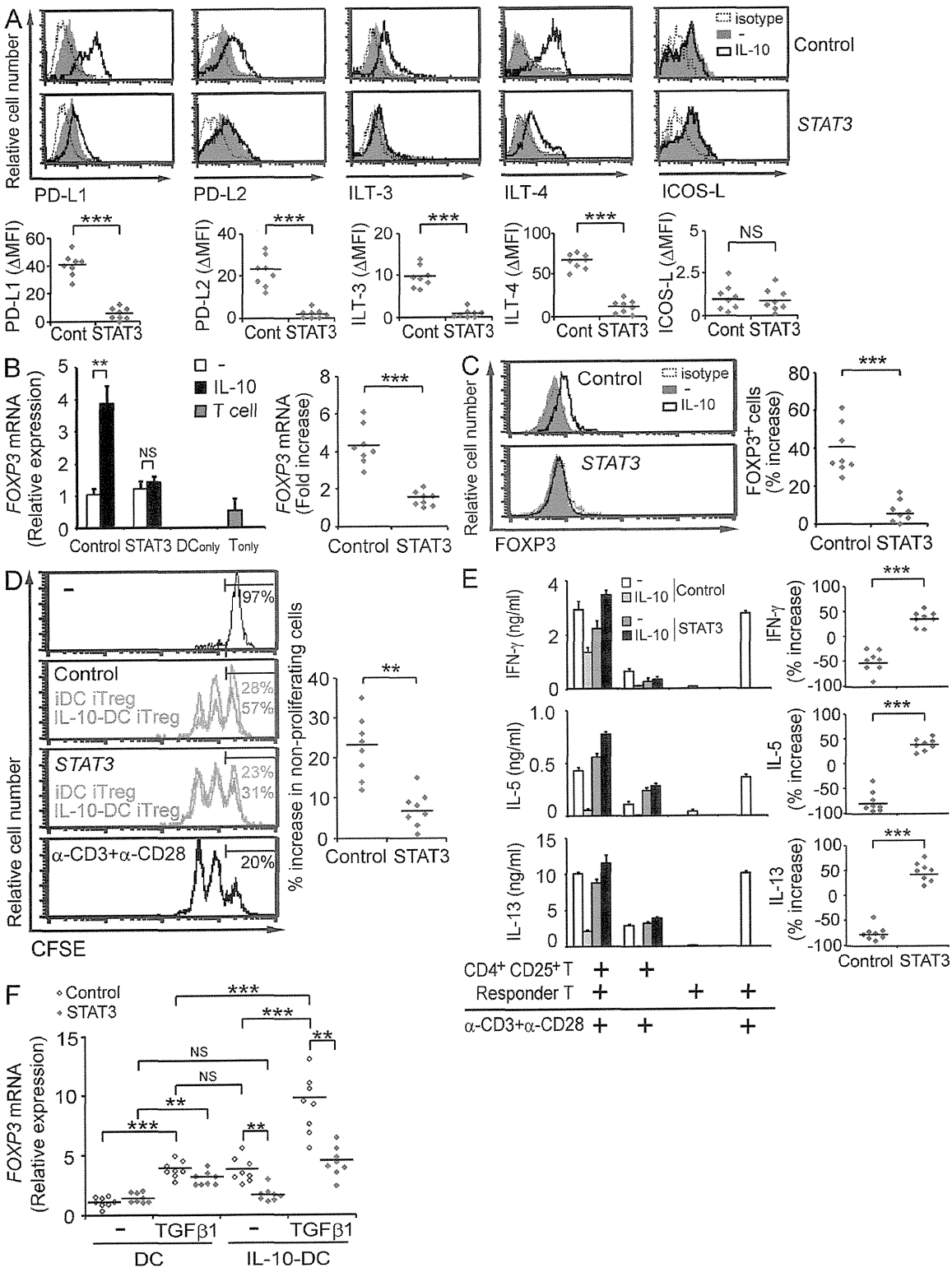

Figure 3. IL-10 signaling defect in MoDCs leads to the defective generation of tolerogenic DCs and iT_{reg} cells. (A) Representative histograms showing the levels of PD-L1, PD-L2, ILT-3, ILT-4, and ICOS-L produced by untreated immature MoDCs (–) and IL-10–DCs (IL-10) from a control subject and a *STAT3*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

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. **, $P < 0.01$; ***, $P < 0.001$.

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

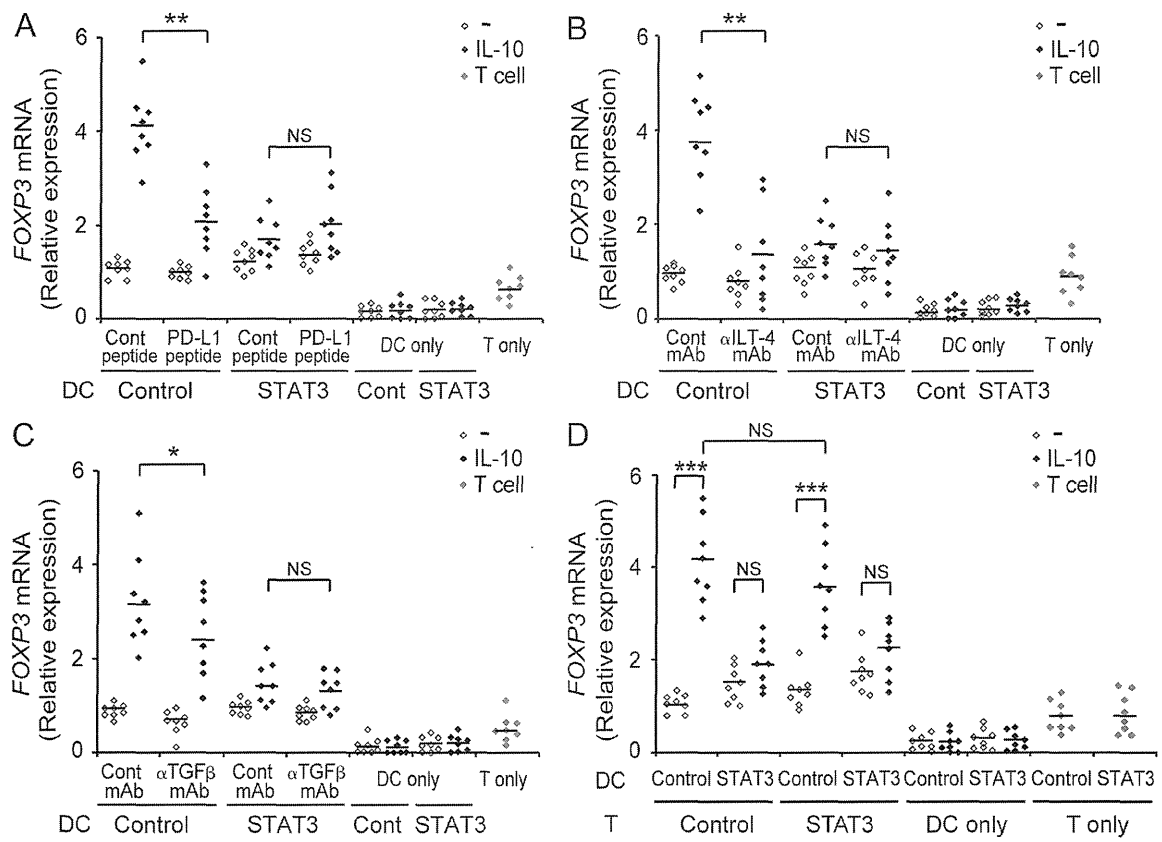

Figure 4. PD-L1, ILT-4, and TGF-β1 in response to IL-10–DCs and *STAT3* in DCs play a major role in FOXP3 up-regulation. (A–C) Q-PCR analysis of *FOXP3* mRNA levels in third-party allogeneic naive $CD4^+$ T cells from control (Cont) subjects co-cultured with untreated immature MoDCs (−) and IL-10–DCs (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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(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.

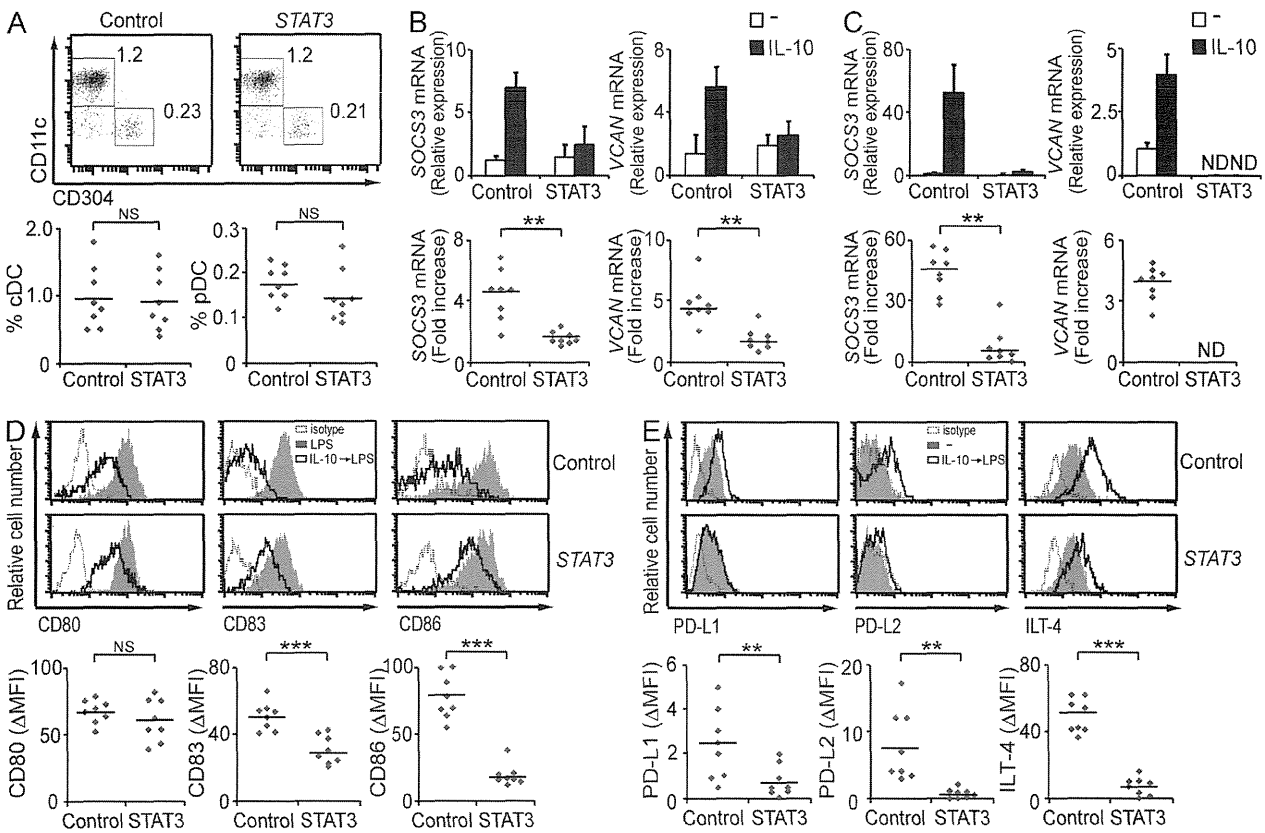

Figure 5. Primary DCs are defective in IL-10 signal and up-regulation of PD-L1 and ILT-4. (A) Dot blots are gated on Lin-negative HLA-DR-positive cells from a control subject and a *STAT3* patient. cDCs are $CD11c^+CD304^-$ (BDCA-4), and pDCs are $CD11c^-CD304^+$. Representative dot plots from a control 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. **, $P < 0.01$; ***, $P < 0.001$.

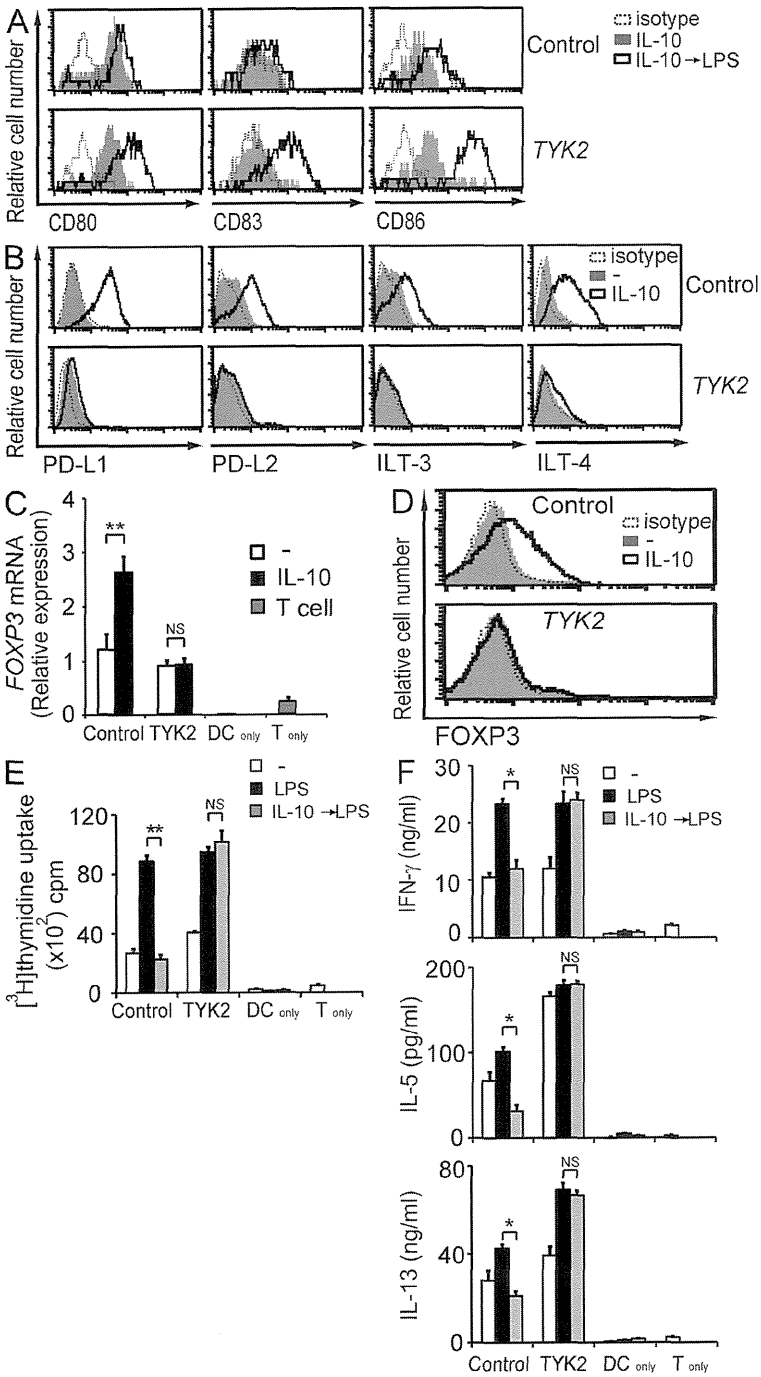

Figure 6. *TYK2*-deficient MoDCs display defective generation of tolerogenic DCs and iT_{reg} cells. (A) Flow cytometric analysis of the CD80, CD83, and CD86 expression on IL-10–DCs (IL-10) and LPS-matured MoDCs after prior treatment with IL-10 (IL-10 → LPS), with cells obtained from a control subject 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

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

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.

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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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

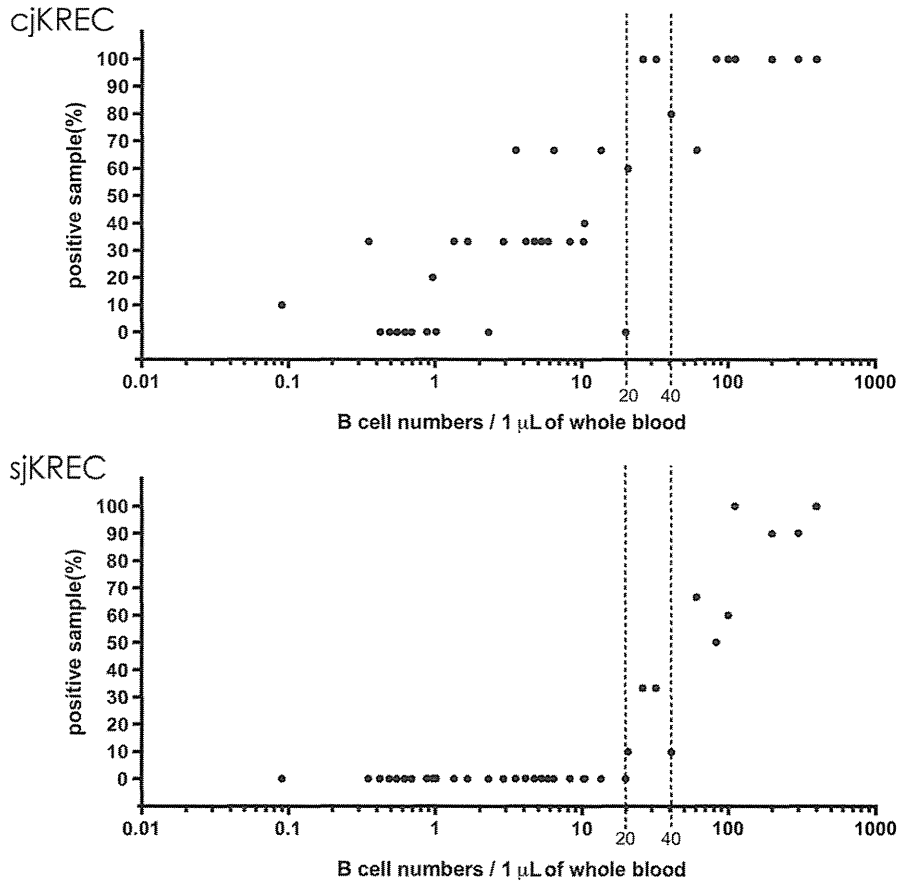

FIG 1. Sensitivity levels of cjKRECs and sjKRECs. Various numbers of purified normal B cells were serially added to whole PB from a patient with XLA (P20) to obtain B-cell–added XLA whole blood. cjKRECs and sjKRECs were measured in 3 to 10 samples of each concentration in triplicate. In all analyses, RNaseP (internal control) was positive ($2.3 \pm 0.2 \times 10^5$ copies/μg DNA). *X-axis,* B-cell numbers in 1 μL whole blood from a patient with XLA. *Y-axis,* Percentages of the KREC-positive results in the tests.

as the B-cell concentrations increased (Fig 1). None of the samples were positive for sjKRECs when the B-cell numbers were less than 20/μL, but cjKRECs were often positive. It has been reported that 90% of patients with XLA have less than 0.2% B cells in the PB at diagnosis.[1] Because peripheral lymphocyte numbers in neonates range from 1200 to 9800/μL,[8] the absolute B-cell numbers of 90% of patients with XLA are estimated to be 2.4 to 19.6/μL at the time of blood collection for Guthrie cards, although exact B-cell numbers of XLA in neonatal periods are not known at this moment. Because neonates are known to have fewer B cells than infants,[9] and we observed that B-cell numbers are constantly low in patients with XLA throughout infancy (Nakagawa, unpublished data, June 2010), which is consistent with the fact that BTK plays an essential role in B-cell maturation. It is likely that neonates with XLA also have severely decreased B cells. On the other hand, all samples obtained from 400 B cells/μL were positive for both cjKRECs and sjKRECs. We also observed that all healthy infants (1-11 months old; n = 15) were sjKREC-positive (Nakagawa, unpublished data, June 2010) and might have at least 600 B cells/μL whole blood.[9] From these data, it is assumed that at least 90% of patients with XLA are sjKREC-negative, and healthy neonates are positive for sjKRECs on neonatal Guthrie cards.

Next, we measured cjKRECs and sjKRECs in dried blood spots in filter papers or Guthrie cards from 30 patients with XLA and 5 patients with non-XLA and from 133 neonates born at the National Defense Medical College Hospital during this study period (August 2008 to October 2009) and 138 healthy subjects of various ages (1 month to 35 years old) to investigate the validity of this method. The levels of B cells of the patients ranged from 0.0% to 1.1% of total lymphocytes and 0.0 to 35.78/μL. IgG levels were 10 to 462 mg/dL (see this article's Tables E1 and E2 in the Online Repository at www.jacionline.org). Patients with leaky phenotypes[1,10] were included; 1 patient (P30) had more than 1% B cells and 34.22/μL total B cells, and 4 patients had more than 300 mg/dL serum IgG (P12, P30, P31, P33). All of the normal neonatal Guthrie cards were positive for both cjKRECs and sjKRECs ($7.2 \pm 0.7 \times 10^3$ and $4.8 \pm 0.6 \times 10^3$ copies/μg DNA, respectively). All healthy subjects of various ages were also positive for both cjKRECs and sjKRECs (Nakagawa, unpublished data, June 2010). In contrast, specimens from all 35 B-cell–deficient patients were sjKREC-negative ($<1.0 \times 10^2$ copies/μg DNA; Fig 2). All 5 patients with leaky phenotypes were also sjKREC-negative, which might be explained by the hypothesis that leaky B cells of patients with XLA are long-lived B cells that divided several times and have fewer sjKRECs than naive B cells.

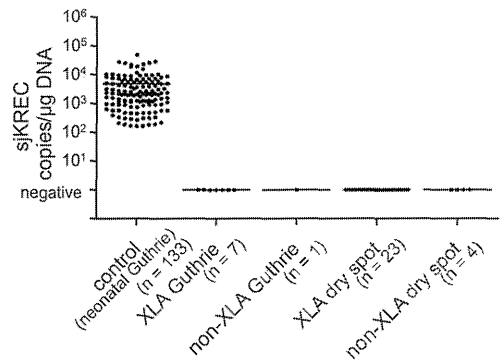

FIG 2. Copy numbers of sjKRECs measured in neonatal Guthrie cards or dried blood spots obtained from B-cell–deficient patients. On all samples from control, neonatal Guthrie cards (n = 133) were sjKREC-positive (4.8 ± 0.6 × 10^3 copies/μg DNA). B-cell–deficient patients were negative for sjKRECs in neonatal Guthrie cards (XLA, n = 7; non-XLA, n = 1) and dried blood spots (XLA, n = 23; non-XLA, n = 4).

One patient (P27) was positive for cjKRECs, but other patients were negative for it. *RPPH1* (internal control) was detectable at the same level as in normal controls in all samples.

These results indicate that sjKRECs are undetectable in XLA and non-XLA and suggest that measurement of sjKRECs in neonatal Guthrie cards has the potential for the use of newborn mass screening to identify neonates with early B-cell maturation defects. Greater numbers of neonatal Guthrie cards should be examined to confirm this potential, and the data obtained from dried blood spots on filter papers must be examined to prove that they truly reflect the data obtained from neonatal Guthrie cards. We should also examine whether screening can reduce the cost of treatment of the bacterial infections and chronic lung diseases in patients with XLA and non-XLA and increase the benefits for these patients. An anticipated pilot study using a large cohort of newborns must address these problems. We also found that T-cell–receptor excision circles and sjKRECs can be measured simultaneously on the same plate. Thus, a pilot study of neonatal screening for both T-cell and B-cell deficiencies could be performed simultaneously.

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doi:10.1016/j.jaci.2011.01.052

TABLE E1. Characteristics of patients with XLA

Patient no.	Unique patient no.	Age (y)	Sex	Serum Ig (mg/dL) IgG	IgA	IgM	$CD19^+$ % Lymph	$CD19^+$ /μL	*BTK* mutation Genomic DNA	cDNA	Amino acid	Source Guthrie	Dry spot
P1	670	0	M	87	<6	10	0.21	12.99	29269G>T	1178-1G>T	Splice acceptor defect	x	
P2	718	0	M	215	<10	<10	0.07	7.04	11593_11594 insA	144_145insA	Arg49 frameshift	x	
P3	722	0	M	80	<1	1	<1.00	NA	25644C>T	763C>T	Arg255X	x	
P4	727	8	M	295	59	57	0.11	3.52	29269G>T	1178-1G>T	Splice acceptor defect		x
P5	732	34	M	1140*	<6	8	0.02	0.24	11631T>A	182T>A	Ile61Asn		x
P6	811	24	M	458*	0	13	0.50	5.32	23570T>G	426T>G	Tyr142X		x
P7	813	18	M	628*	109	6	0.60	6.87	23570T>G	426T>G	Tyr142X		x
P8	814	19	M	260	0	NA	0.20	3.01	16180C>T	344C>T	Ser115Phe		x
P9	815	13	M	600*	<10	<5	0.08	1.72	11590G>T	142-1G>T	Splice acceptor defect		x
P10	816	11	M	12	0	5	0.00	0.00	150kb deletion of *BTK, TIMM8A, TAF7L, DRP2*				x
P11	817	10	M	10	2	24	0.80	35.78	36288C>T	1928C>T	Thr643Ile		x
P12	824	13	M	462	6	27	0.41	14.49	27518C>A	895-11C>A	Splice acceptor defect		x
P13	834	5	M	<237	<37	43	0.00	0.00	25715_26210del	776+57_839+73del	Exon 9 deletion		x
P14	838	21	M	<50	<5	7	0.00	0.00	31596G>C	1631+1G>C	Splice donor defect		x
P15	839	16	M	604*	<1	<2	0.04	0.66	31596G>C	1631+1G>C	Splice donor defect		x
P16	847	11	M	698*	26	11	0.08	1.86	25536delG	655delG	Val219 frameshift		x
P17	877	14	M	20	19	8	0.21	NA	32357T>C	1750+2T>C	Splice donor defect		x
P18	880	5	M	233	39	41	0.06	NA	10941-?_14592+?del	1-?_240+?del	Exon 1-3 deletion		x
P19	888	8	M	<212	<37	150	0.15	6.60	11023G>A	83G>A	Arg28His		x
P20	891	21	M	195	<6	37	0.02	0.09	32243C>G	1638C>G	Cys502Trp		x
P21	958	0	M	<50	<10	9	0.80	27.14	31544_31547 delGTTT	1580_1583del GTTT	Cys527 frameshift		x
P22	701	2	M	115	<2	4	0.09	1.99	16172C>A	336C>A	Tyr112X		x
P23	911	0	M	<10	<6	<4	0.00	0.00	29955A>C	1350-2A>C	Splice acceptor defect	x	
P24	937	0	M	60	<2	58	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P25	938	0	M	<20	<4	<6	0.00	0.00	36269-?_36778+?del	1909-?_2418+?del	Exon 19 deletion	x	
P26	939	0	M	60	<2	22	0.00	0.00	11022C>T	82C>T	Arg28Cys	x	
P27	890	12	M	<237	<37	<20	0.03	NA	36261G>A	1909-8G>A	Splice acceptor defect		x
P28	944	6	M	12	<1	1	0.02	NA	36281C>T	1921C>T	Arg641Cys		x
P29	948	5	M	<237	<37	<20	0.01	0.70	36261G>A	1909-8G>A	Splice acceptor defect		x
P30	1053	5	M	386	5	113	1.10	34.22	32259A>C	1654A>C	Thr552Pro		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *M,* male; *NA,* not available; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

BTK mutation's reference sequences are NCBI NC_000023.9, NM_000061.2, and NP_000052.1.

*Trough level during intravenous immunoglobulin therapy.

TABLE E2. Characteristics of patients with non-XLA

				Serum Ig (mg/dL)			$CD19^+$			Source	
Patient no.	Unique patient no.	Age (y)	Sex	IgG	IgA	IgM	% Lymph	/μL	*BTK* mutation	Guthrie	Dry spot
P31	596	4	F	386	<6	6	0.42	21.27	Normal		x
P32	719	0	F	<50	<5	<5	0.00	0.00	Normal	x	
P33	835	8	M	311	323	20	0.09	1.88	Normal		x
P34	915	0	M	<212	<37	<20	0.00	0.00	Normal		x
P35	947	0	M	<21	<37	<39	0.00	0.00	Normal		x

Age, Age at analysis of KRECs; *$CD19^+$ % Lymph,* CD19-positive cell percentage in lymphocytes; *$CD19^+$ /μL,* CD19-positive cell number in 1 μL whole peripheral blood; *F,* female; *M,* male; *Serum Ig,* serum levels of immunoglobulins at diagnosis.

Brief report

Autoimmune lymphoproliferative syndrome–like disease with somatic *KRAS* mutation

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Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (type I-III). Germline *NRAS* mutation was recently identified in type IV ALPS. We report 2 cases with ALPS-like disease with somatic *KRAS* mutation. Both cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia and are probably defined as a new disease entity of RAS-associated ALPS-like disease (RALD). (*Blood*. 2011;117(10):2887-2890)

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway,[1,2] currently categorized as: type Ia, germline *TNFRSF6/FAS* mutation; type Ib, germline *FAS ligand* mutation; type Is, somatic *TNFRSF6/FAS* mutation; and type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases, such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes types III and IV, whereby type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis and type IV as one showing germline *NRAS* mutation.[3] Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to interleukin-2 (IL-2) depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS-associated leukoproliferative disease.[4]

Juvenile myelomonocytic leukemia (JMML) is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. Approximately 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells, including mutations of *NF1*, *RAS* family,[5] *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood mononuclear cells (MNCs) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of $CD34^+$ BM-MNCs.[6]

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7]; among these tumors, the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present 2 cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic KRAS G13D mutation without any clinical features of germline *RAS* mutation, such as cardio-facio-cutaneous or Noonan syndrome.

Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (supplemental Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Blood test revealed the presence of hemolytic anemia and autoimmune thrombocytopenia. Hyper-γ-globulinemia with various autoantibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in "Results and discussion."

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