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Defective CTLA-4 cycling pathway in Chediak–Higashi syndrome: A possible mechanism for deregulation of T lymphocyte activation

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ABSTRACT **Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, also known as CD152) has been shown to play a major role in the regulation of T cell activation. Its membrane expression is highly regulated by endocytosis and trafficking through the secretory lysosome pathway. Chediak–Higashi syndrome (CHS) is an inherited disorder caused by mutations in the lysosomal trafficking regulator gene, *LYST*. It results in defective membrane targeting of the proteins present in secretory lysosomes, and it is associated with a variety of features, including a lymphoproliferative syndrome with hemophagocytosis. The murine equivalent of CHS, *beige* mice, present similar characteristics but do not develop the lymphoproliferative syndrome. We show herein that CTLA-4 is present in enlarged, abnormal vesicles in CHS T cells and is not properly expressed at the cell surface after T cell activation, whereas its surface expression is not impaired. It is therefore proposed that the defective surface expression of CTLA-4 by CHS T cells is involved in the generation of lymphoproliferative disease. This observation may provide insight into the role of CTLA-4 in humans.**

The maintenance of the homeostatic balance in immune regulation has been the focus of a number of recent studies. T cell activation results from the integration of signals generated through the T cell receptor (TCR) with those from additional positive and negative regulatory pathways (1, 2). Disruption of this balance leads to a defective immune response (3–5) or, alternatively, over-activation of the immune system (6–8), as observed in several genetically determined human diseases. Fatal, uncontrolled T cell activation (also called the "accelerated phase"), is a feature of a rare, autosomal, recessive human disease, the Chediak–Higashi syndrome (CHS) (9). Spontaneously or, more usually, after a viral infection, CHS patients present with accumulation of nonmalignant, activated T lymphocytes, mostly $CD8^+$ and macrophages (typically with hemophagocytosis) in several organs (10). This unusual lymphocyte and macrophage activation can be treated successfully with lympho-ablative reagents (anti-thymocyte globulins) or with immunosuppressive drugs such as cyclosporin A (11), which suggests that the defective control of T cell homeostasis is primarily responsible for this syndrome.

The defective gene in CHS has been identified as the lysosomal trafficking regulator (*LYST*) (12, 13). This gene has limited structural similarity to the yeast phosphatidylinositol 3-kinase VPS15 that is involved in protein sorting. The detected human LYST product has been associated with microtubules and is involved in the sorting of MHC class II molecules and other proteins to late multivesicular compartments in B cells (14). The precise function of LYST is unknown, but the available data strongly suggest that this protein is involved in regulating the secretory processes of intracellular lysosomal vesicles.

A similar genetic disorder has also been described in *beige* mice. However, in contrast to what is observed in almost all of the CHS patients, the development of a lymphoproliferative syndrome has not been reported in any variety of known *beige* mutant mice, even after challenge with infection (15–17). CHS patients and *beige* mice, however, share the other features of this syndrome, including abnormal skin and hair pigmentation and a cytotoxic functional defect in T and natural killer (NK) cells (18–20). These features probably result from a block in the secretory lysosomal pathway. Abnormal giant granules are a typical feature of CHS patients and *beige* mice, and are observed in many cell types engaged in controlled secretory processes (9). These enlarged granules are lysosome-like, secretory fusion structures (21). They store melanin in melanocytes (9) and retain granzymes and perforin lytic proteins in cytotoxic T and NK cells, which defines them as secretory lysosomes (19, 20). It has been proposed that a defect in the secretion of these enlarged vesicles may be associated with the phenotype. The uncontrolled activation of T lymphocytes in CHS may thus result from impaired sorting and secretory activity and the subsequent expression of immune regulatory molecules.

An attractive candidate for a T cell activation regulator is the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (22). There is accumulating evidence that CTLA-4 acts as a negative regulator of T cell activation and is involved in termination of the T cell response (23, 24). Blocking of CTLA-4 *in vivo* has been shown to increase antitumor immunity (25) and exacerbate autoimmune disease (26, 27). The key role of CTLA-4 in maintaining homeostasis in the immune system has been clearly illustrated by genetically deficient $CTLA\text{-}4^{-/-}$ mice (6, 7), which develop a fatal lymphoproliferative disorder reminiscent of the lymphoproliferative syndrome observed in human CHS. Subtle regulation of CTLA-4 membrane expression is required to orient immune response to either T cell activation or tolerance induction. Indeed, although CTLA-4 has the characteristics and ligand-binding properties of plasma membrane receptors, it is found mainly in intracellular vesicles, i.e., in the endocytic compartments and secretory granules (28, 29). It recirculates between intracellular stores and the cell surface

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: CHS, Chediak–Higashi syndrome, CTLA-4, cytotoxic T lymphocyte-associated antigen 4; LYST, lysosomal trafficking regulator; PBMCs, peripheral blood mononuclear cells; TCR, T cell receptor.

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through its association with the $\mu 2$ subunit of the adapter complex, AP-2 (30–32). Endocytosis and signaling are both regulated by tyrosine phosphorylation of CTLA-4 cytoplasmic tail and require a polarized export to the site of TCR engagement (23, 24, 30–32). These factors suggest that TCR-induced tyrosine kinases are involved in determining the state of CTLA-4 phosphorylation (33, 34). Therefore, defects in the trafficking of CTLA-4-membrane export are likely to disturb T cell homeostasis significantly.

In this study, we show that intracellular trafficking of CTLA-4 is impaired in the T cells of CHS patients and results in defective cell-surface expression of this molecule. In contrast, little is defective in CTLA-4 trafficking in *beige* mouse T cells, and membrane expression of CTLA-4 is normal. Our hypothesis, therefore, is that defective CTLA-4 membrane expression occurs with the lymphoproliferative syndrome in human CHS, but it does not occur in the murine model of the disease.

MATERIALS AND METHODS

Patients. The three patients with CHS who are reported in this study presented with the characteristic features of the syndrome. Patient 1 and patient 2 developed the hemophagocytic lymphoproliferative syndrome at the ages of 16 months and 12 years, respectively. The syndrome caused the death of patient 1, whereas it was arrested in patient 2 after treatment with steroids and cyclosporin A. An investigation of leukocytes was performed on patient 1 during the course of the lymphoproliferative syndrome, before lymphocyte ablative treatment, and on patient 2, after 6 months to 2 years of continuous remission. Patient 3, 17 years old, had not developed an accelerated phase, but presented the classical phenotype of CHS, including albinism and the natural killer cell defect associated with the presence of enlarged granules. In these patients, as in other patients previously investigated at our center, the lymphoproliferative syndrome was characterized by an increase in the number of peripheral T lymphocytes, marked hepatosplenomegaly, polyadenopathy, and the infiltration of various organs, including the brain, by activated T lymphocytes and macrophages (35). In patient 1, two different heterozygous mutations were detected, both leading to a stop codon (positions 1785 and 3210). In patient 2, the mutation in the *LYST* gene was a homozygous deletion at nucleotide 7748, with the resulting frame shift creating a stop codon at position 2528 (S. Certain, F.J.B., E. Pastural, F.L.D., J. Goyó-Rives, N. Jabado, M. B., R. Seper, E. Vilmer, G. Beullier, D. Schwarz, A.F., and G.d.S.B., unpublished data). In patient 3, the mutations are still undetermined. All investigations have been performed after informed consent and conducted according to the principles expressed in the Declaration of Helsinki.

Mice. The strains of mice used in this study were C57BL/6 and *beige* C57BL/6-*bg*^{J}, the latter the result of a spontaneous mutation in strain C57BL/6 at the Jackson Laboratory (36).

mAbs. Anti-CD3 (UCHT1), anti-CD28, anti-CD63, and biotinylated rat anti-mouse mAbs were purchased from Immunotech (Marseille, France). Anti-CTLA-4 (BNI3.1, IgG2a), and control anti-TNP (IgG2a) mAbs, anti-mouse CD3ε, anti-mouse L-selectin CD62L (Mel14), anti-mouse lysosome-associated proteins Lamp-1 and Lamp-2, hamster anti-mouse CTLA-4 (UC10–4F10–11), and mouse anti-hamster IgG mAbs were purchased from PharMingen (San Diego). Anti-perforin mAb dG9 was purchased from Ancell (Bayport, MN). Texas red-coupled goat anti-mouse IgG2a was purchased from Southern Biotechnology (Birmingham, AL). Phycoerythrin-coupled streptavidin and rhodamine-coupled streptavidin were purchased from Caltag (South San Francisco, CA).

Immunofluorescence and Confocal Microscopy. Cells were washed twice and fixed in PBS containing 3.7% paraformaldehyde and 30 mM sucrose for 15 min at 4°C and then incubated for 10 min at room temperature in 50 mM NH_4Cl/PBS. The cells were then incubated in permeabilizing buffer (PBS/BSA containing 0.05% saponin) with the appropriate antibodies for 45 min at 4°C. The cells were washed twice in PBS/BSA/saponin before staining with the second mAb. After being stained, the cells were mounted on microscope slides and examined under a confocal microscope (Leica).

Flow Cytometry. Cells were washed twice in ice-cold PBS/0.05% sodium azide and stained with saturating concentrations of the appropriate antibody. CTLA-4 surface staining in human cells was performed by indirect immunofluorescence with a biotinylated rat anti-mouse antibody (revealed with phycoerythrin-coupled streptavidin). For intracellular staining, the cells were fixed and permeabilized before being stained as described above. To avoid staining of surface CTLA-4 molecules, noncoupled anti-CTLA-4 mAb was added before cell fixation. Ten thousand viable cells were measured with a FACScan flow cytometer (Becton Dickinson) and analyzed with the CELLQUEST or LYSIS II software packages.

T Cell Repertoire Analysis. TCR V_β family usage by the patient's T cell population was analyzed with the immunoscope-based technique as previously described (37, 38).

Cell Culture. Human peripheral blood mononuclear cells (PBMCs) were isolated with standard procedures and were activated by incubation on anti-CD3 (500 ng/ml) and anti-CD28 (2 μg/ml) mAb-coated chambered coverslips (Nunc) for 2 days. For allogeneic activation, cells (1×10^5 per well) were cultured with a mixture of three different γ-irradiated (6000 rads) Epstein–Barr virus-transformed B cell lines (30×10^4 cells per well) for 3 days. In some experiments, the cells were incubated for 10 min at 37°C with a freshly prepared solution of pervanadate. A stock solution (50×) was made with 500 mM H_2O_2 and 5 mM Na_3VO_4 and left to stand for 15 min at room temperature before being used.

Mouse lymph node cells were prepared with standard procedures and activated by incubation with an anti-CD3ε mAb (10 μg/ml) for 2 to 3 days. For allogeneic activation, the lymph node cells were plated (10^6cells per well) and incubated for 96 h with γ-irradiated (2000 rads) spleen cells prepared from DBA/2 mice (4×10^6 cells per well).

RESULTS

The Lymphoproliferative Syndrome in CHS Results in the Expansion of Activated CD8$^+$ T Cells. The hemophagocytic–lymphohistiocytic syndrome observed in CHS patients (see *Materials and Methods*) results in an approximately 2- to 10-fold increase in the number of peripheral T lymphocytes (Table 1). T cells were shown to exhibit activation markers, i.e., HLA class II and CD95/Fas antigen (Table 2). Other activation markers, CD25 (IL-2 Rα), CD40 ligand (CD154), and CD69, were barely or inconstantly detected on these T cells, possibly because of the lability of these molecules during prolonged lymphocyte activation (Table 2). This lymphoproliferation was found to be polyclonal, as shown by immunoscope analysis of the T cell repertoire of patient 1, because no TCR V_β family was predominant (data not shown). Both CD4$^+$ and CD8$^+$ T lymphocytes were found to be activated, but most

Table 1. Phenotype analysis of CHS patients' lymphocytes: CD3, CD4, and CD8

	Patient 1	Patient 2	Patient 3	Control
Number/mm^3	14,200	3,000	2,500	2,000–5,000
CD3, %	79	76	69	70–90
CD4, %	8	50	31	60–80
CD8, %	69	27	39	20–40

Patient 1 was studied at time of lymphoproliferative syndrome onset. Patient 2 was studied 2 years after remission of lymphoproliferative syndrome. Patient 3 had not developed lymphoproliferative syndrome.

Table 2. Phenotype analysis of CHS patients' lymphocytes: Further analysis of $CD3^+$, $CD4^+$, and $CD8^+$ cells

	Patient 1			Patient 2	Patient 3	Control
Antigen	CD3	CD4	CD8	CD3*	CD3*	CD3*
HLA-DR, %	75	66	80	7	14	<15
CD25, %	2	4	0	2	5	<10
CD69, %	0	0	0	1	0	<5
CD28, %	84	99	80	80	92	>80
CD95,† %	70‡	61‡	73‡	59	68	30–60
CD45RO, %	65	54	66	44	59	40–60
CD45RA, %	30	38	25	56	34	40–60
CD40L, %	0	0	0	1	0	<5

Patients were studied at the times described for Table 1.
*The results were similar for CD4 and CD8 cells.
†Low expression.
‡Age-matched control: <30%.

of the peripheral T cells, as well as the T lymphocyte population infiltrating the various organs of the CHS patients, were $CD8^+$ cells (35) (Table 2). There was no excess of activated $CD8^+$ T cells in patients 2 and 3 tested while not suffering from the lymphoproliferative syndrome (Table 1).

Intracellular CTLA-4 Is Located in the Giant Granules of T Lymphocytes in CHS patients and *beige* Mice. Confirming a previous description (29), we found that a large fraction of the internal store of CTLA-4 molecules are present in perforin-labeled vesicles (Fig. 1 *A–C*). These vesicles, known as secretory lysosomes (39), may direct the release of CTLA-4 molecules to the site of activation (29). Although in CHS cytotoxic T cells intracellular CTLA-4 stores displayed the same location as perforin, we showed that CTLA-4 was exclusively contained in the enlarged abnormal vesicles frequently identified as unique giant granules in each perforin-positive cell (Fig. 1 *E–G*). In $CD4^+$ cells, CD63, a lysosomal membrane protein with four membrane-spanning domains, was used as a lysosome-specific marker. Intracellular CTLA-4 was found not only in the giant granules labeled with CD63, but also in smaller $CD63^+$- or $CD63^-$-labeled vesicles (not shown) that are probably part of the endocytic pathway.

When directional polarization of CTLA-4 was analyzed, it was observed that the large CTLA-4-containing vesicles tended to concentrate near the plane of the coverslips in CHS T cells (Fig. 1*H*), in a manner similar to what is observed in control cells (Fig. 1*D*). Thus, the intracellular CTLA-4 store in CHS cells is found mostly in the abnormally enlarged granules and exclusively so in $CD8^+$/perforin-positive cells.

In mouse T cells, the intracellular CTLA-4 store was contained in lysosomal structures identified by anti-Lamp-1 (Fig. 2) or anti-Lamp-2 (data not shown) antibodies. Some of these structures were larger in the *beige* mutant than in the control mice, but none were as large or were fused to a single granule, as is observed in CHS cells (Fig. 2 *D–F* vs. Fig. 1*E*). Moreover,

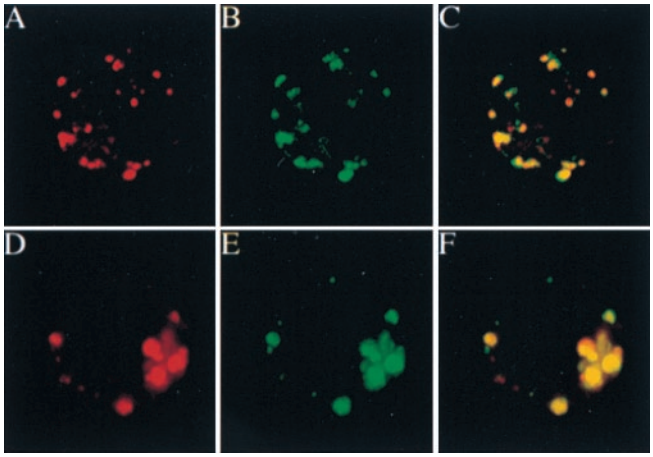

FIG. 2. Intracellular expression pattern of CTLA-4 in activated T cells of C57BL/6 and *beige* mice. T cells isolated from the lymph nodes of C57BL/6 (*A–C*) and *beige* (*D–F*) mice were activated for 48 h with an anti-CD3ε mAb (10 μg/ml), fixed, permeabilized, and stained for CTLA-4 (*A, D*) and FITC-coupled anti-Lamp-1 (*B, E*) mAbs. The overlapping distribution between CTLA-4 and Lamp-1 is shown in yellow (*C, F*).

some CTLA-4 molecules were found not to be contained inside these enlarged granules, suggesting that a subset of CTLA-4 may not be affected in the *beige* mouse T cells. Assessment of CTLA-4 detection in lytic granules was not feasible because of poor intracellular staining with mouse anti-perforin antibodies.

Defective Cell Surface Expression of CTLA-4 by T Lymphocytes in CHS. It has been shown that lytic enzymes are not properly released from the intracellular stores of CHS CTL clones (20). We therefore investigated the possibility that CTLA-4 expression at the surface of $CD8^+$ CHS cells was also defective. Whereas activated control lymphocytes showed a polarized surface expression of CTLA-4 toward the site of TCR-mediated activation (Fig. 3*A*), CTLA-4 surface expression was not detected on activated T cells from patient 1, who was studied during the course of the lymphoproliferative syndrome (Fig. 3*B*). Prolongation of the activation time to 72 h did not restore surface CTLA-4 expression.

A similar study was performed after T cell activation by allogeneic cells. In contrast to what was observed in the control cells, CTLA-4 surface expression was not detectable on the T lymphocytes of CHS patient 1 (Fig. 4*A*), even after pervanadate treatment, which is known to increase CTLA-4 surface expression (31).

In patients 2 and 3, tested while not suffering from the lymphoproliferative syndrome, the abnormal intracellular pattern of CTLA-4 distribution was found to be restricted to the $CD8^+$ T cells, the main lymphoproliferative subset in CHS. As

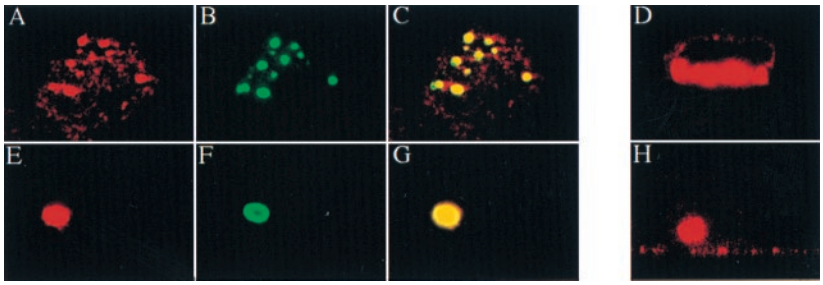

FIG. 1. Detection of CTLA-4 in perforin-containing vesicles and polarization toward sites of T cell activation. PMBC from control (*A–D*) and CHS patient 1 (*E–H*) were activated by incubation on glass coverslips coated with anti-CD3 and anti-CD28 mAbs for 48 h. They were fixed, permeabilized, and stained for CTLA-4 (*A, D, E, H*) (detected by Texas red-coupled goat anti-mouse IgG2a) and FITC-coupled anti-perforin (*B, F*) mAbs. Immunofluorescence was examined by confocal microscopy. The overlapping distribution is shown in yellow (*C, G*). Vertical (*X, Z*) optical sections are shown (*D, H*). Similar results were observed with patient 2 and patient 3.

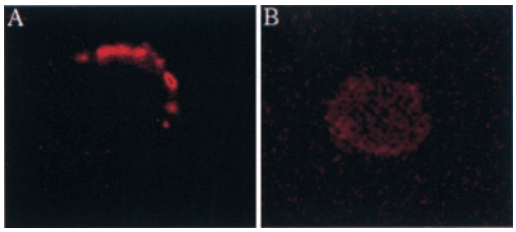

FIG. 3. CTLA-4 surface expression on activated T cells from CHS patient 1 and control T cells. T cells from control (*A*) and CHS patient 1 (*B*) were activated by incubation on glass coverslips coated with anti-CD3 and anti-CD28 mAb for 48 h. They were stained for CTLA-4 and then fixed to detect the surface expression of CTLA-4. Confocal analysis showed that CTLA-4 molecules were polarized at the cell surface in the control, whereas they were undetectable in cells from CHS patient 1. After activation, more than 90% of patient and control cells were $CD69^+$.

shown in Fig. 4*B*, $CD8^+$ T cells from these two different patients were unable to express CTLA-4 after allogeneic activation, even after treatment with pervanadate, whereas CD69 and HLA-DR were detected and were demonstrating a normal allogeneic response. These data suggest that the CTLA-4 defect in CHS is mostly CD8-restricted. However, it was not possible to determine whether the $CD4^+$ population was also affected during the lymphoproliferation, because of the small number of cells collected (patient 1 was 2 years old at the time of the study) and the low percentage of $CD4^+$ T cells (Table 1).

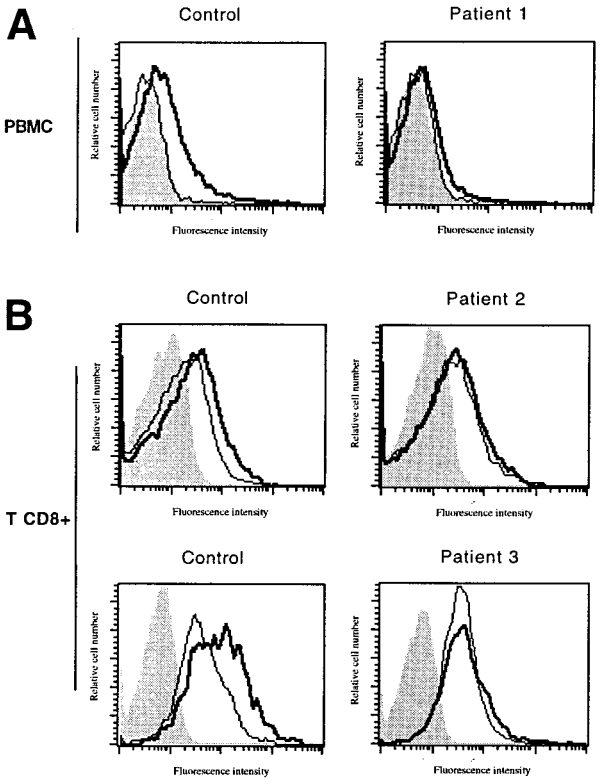

FIG. 4. CTLA-4 surface expression on CHS and control cells after allogeneic activation. (*A*) PBMCs isolated from patient 1 with lymphoproliferative syndrome and control PBMCs were stained for CTLA-4 (thick lines) after 72 h of allogeneic cell stimulation and pervanadate treatment. More than 90% of the cells from patient 1 and the control were $CD69^+$. (*B*) PBMCs isolated from patients 2 and 3 and control PBMCs were co-stained for CD8 and CTLA-4 (thick lines). In both patients and control, more than 75% of the $CD8^+$ T cells expressed CD69 and HLA-DR after activation. Control staining was carried out with an isotype-matched irrelevant mAb (thin lines). Shaded areas represent unstained cells.

Normal Expression of CTLA-4 by the Lymphocytes of *beige* Mice. *beige* mice, the murine homologue of CHS, do not develop a lymphoproliferative syndrome regardless of the *LYST* mutations involved, including the mutations that result in the complete absence of the LYST product (40), and even after a potent infection *in vivo* (15–17). We therefore analyzed the synthesis and expression of CTLA-4 in T lymphocytes from *beige* mice. Intracellular CTLA-4 was detected in activated T cells in both *beige* and control mice (Fig. 5). However, normal CTLA-4 expression at the surface of activated T lymphocytes was detected in *beige* mice (Fig. 5), whereas it was not in the CHS patients. Both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells expressed CTLA-4 normally (data not shown). Moreover, pervanadate treatment of allogeneic activated murine lymphocytes also resulted in an increase of cell-surface expression, in parallel with a decrease in intracellular stores of CTLA-4 molecules in the lymphocytes of *beige* and control mice (Fig. 6). Thus, in contrast to what is observed in human CHS T cells, the intracellular CTLA-4 distribution in *beige* mice was found not to be disturbed enough to interfere with the regulation of its cell-surface expression.

CTLA-4 Is Expressed in CHS but Not in *beige* Mouse T Cells Before Activation. CTLA-4 has been shown to be undetectable in resting control T cells, but its expression increases rapidly after activation (41). Because CHS lymphocytes are activated *in vivo* during the course of the lymphoproliferative syndrome (Table 2), CTLA-4 expression was directly assessed on the T cells of patient 1 before cell activation *in vitro*. CTLA-4 was detected, although detection was restricted to the giant perforin-positive vesicles of the lymphocytes of patient 1. Perforin-negative cells did not express CTLA-4. Furthermore, no CTLA-4 surface expression was detected on lymphocytes from patient 1 pre-activated *in vivo* (data not shown), consistent with the previous observation after *in vitro* activation. As expected, control cells did not express CTLA-4 either within cells or at the membrane. Surprisingly, intracellular CTLA-4 expression was also detected in the perforin-containing giant granules of lymphocytes from patients 2 and 3 (Fig. 7), in the absence of a lymphoproliferative syndrome and *in vitro* cell activation (Table 1). Most (80%) CHS CTLA-4-containing cells expressed the memory T cell marker CD45RO at their surface (data not shown). Treatment with the calcium ionophore ionomycin (29) did not lead to surface expression of CTLA-4 (data not shown). In contrast, no CTLA-4 was detected in resting *beige* mouse T cells, suggesting that perturbation of the CTLA-4 expression pattern differs in the mouse model.

DISCUSSION

The lymphoproliferative syndrome that occurs in CHS is characterized by a polyclonal expansion of T lymphocytes, mostly of the $CD8^+$ phenotype, which then infiltrate various organs. The increase in lymphocyte number in CHS is associated with a defect of the LYST protein, which is involved in intracellular vesicle trafficking and protein transport. Impairment of this process may affect the expression of molecule(s) involved in the induction and maintenance of peripheral tolerance and homeostasis. CTLA-4 is one of the key molecules involved in such regulatory mechanisms, and its expression depends on intracellular vesicle trafficking. The role of this molecule in humans has not been assessed *in vivo*, but polymorphisms in the CTLA-4 gene are associated with Grave's disease and diabetes mellitus (42, 43). In mice, the lymphoproliferative syndrome observed in the $CTLA\text{-}4^{-/-}$ model is reminiscent of the one displayed by CHS patients, although in the latter there is a preferential increase in $CD8^+$ cells (44). Viral infections frequently trigger a lymphoproliferative disorder in CHS patients, and recent studies in mice and humans have demonstrated that viral infections may lead

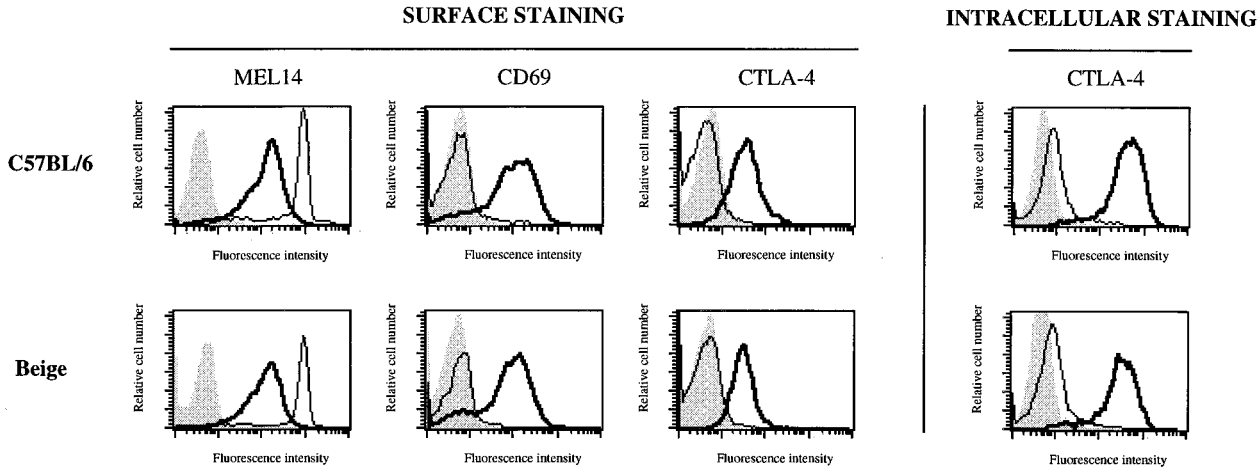

FIG. 5. CTLA-4 surface and intracellular expression by T cells from C57BL/6 and *beige* mice. (*Left*) T cells isolated from the lymph nodes of C57BL/6 and *beige* mice were activated for 48 h with an anti-CD3ε (10 μg/ml) and stained for the CD62L antigen of the L-selectin (Mel14), the activation marker CD69, and the CTLA-4 antigen before (thin lines) and after activation (thick lines). (*Right*) Cells were also stained for intracellular CTLA-4 before (thin lines) and after (thick lines) activation. Shaded areas represent unstained cells.

to a major increase in the number of antigen-specific $CD8^+$ lymphocytes (45).

In this study we show that CTLA-4 is targeted to the abnormal intracellular giant granules in the T cells of CHS patients. CTLA-4 is stored partly within these abnormal structures in $CD4^+$ lymphocytes and exclusively within them in the $CD8^+$ subset of lymphocytes of CHS patients. These vesicles are often found as a single giant granule that also contains the lytic enzymes. In agreement with the finding of the lytic enzyme secretory defect previously described in $CD8^+$ T cells in CHS, CTLA-4 intracellular stores cannot be properly mobilized to the cell surface of CHS T cells after lymphocyte activation. This defect was clearly observed in one patient during the course of the lymphoproliferative syndrome. It was also observed within the $CD8^+$ population in two other patients studied while not suffering from the lymphoproliferative syndrome.

In contrast, in the *beige* mouse, the intracellular localization of CTLA-4 in T cells is not as strongly impaired as it is in the CHS T cells. Indeed, it is possible to detect some CTLA-4 molecules outside the enlarged granules, which may not be affected in these cells (Fig. 2). This observation correlates with that of the normal expression and regulation of CTLA-4 at the cell surface of the T cells in this mutant and may explain why the mouse model of CHS does not have a lymphoproliferative disorder, even under infection or a condition of activation.

During an immune response to an infective agent, the CTLA-4 defect in patients may be overcome by alternative interactions which, however, may not be strong enough if the infection induces a large increase in $CD8^+$ clones, as recently shown in Epstein–Barr viral infections (45). This is supported by the fact that Epstein–Barr virus is most often associated with lymphoproliferative syndrome induction (S. Certain, *et al.*, unpublished data). Furthermore, in CHS, the additional defective cytotoxic activity possibly affecting virus-infected cells may also interfere with the specific immune response of the $CD8^+$ subset.

The fact that the CTLA-4 defect is restricted to the $CD8^+$ subset may be explained by a differential effect of LYST anomalies on CTLA-4 trafficking in the $CD4^+$ and the $CD8^+$ populations, with the LYST anomalies having a greater effect in the $CD8^+$ cells. This notion is supported by the preferential uncontrolled expansion of this cell population during the lymphoproliferative syndrome and the continuous presence of intracellular stores containing CTLA-4 in the CHS $CD8^+$ T cell subset only. This pre-existing intracellular CTLA-4 store in $CD8^+$ T cells may result from a CTLA-4 release defect in the cells previously activated *in vivo*. Defective release should lead to defective surface expression of CTLA-4, and thus to an uncontrolled specific lymphocyte response. The nature of the enlarged granule may also be slightly different in the $CD4^+$ subset from that in the $CD8^+$ subset. Indeed, in the $CD8^+$ cells, these enlarged granules contained perforin and granzymes and are probably devoted to the secretion process, whereas, in the

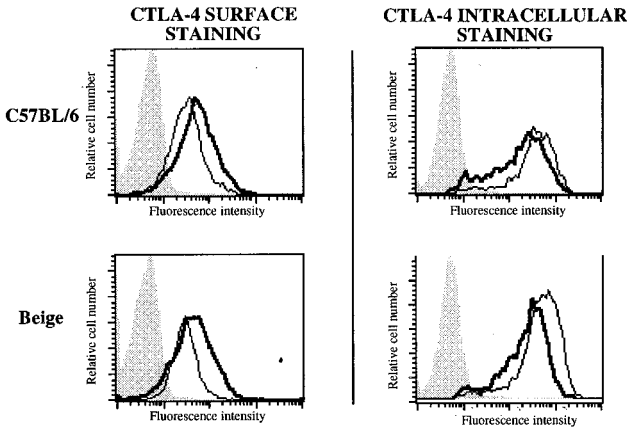

FIG. 6. CTLA-4 surface and intracellular expression of pervanadate-treated T cells from C57BL/6 and *beige* mice. T cells isolated from the lymph nodes of C57BL/6 and *beige* mice were stained for surface and intracellular CTLA-4 after being activated for 96 h with γ-irradiated (2000 rads) spleen cells prepared from DBA/2 mice. Cells were incubated with (thick lines) or without (thin lines) pervanadate.

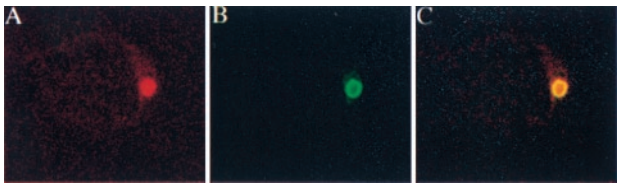

FIG. 7. Intracellular CTLA-4 expression in CHS T cells before *in vitro* cell activation. Nonactivated T cells from CHS patient 2 were fixed, permeabilized, and stained as in Fig. 1 for CTLA-4 (*A*) and perforin (*B*) and examined by confocal microscopy. There was no CTLA-4 staining in control cells (not shown). Staining in cells from patient 2 was associated with perforin-containing vesicles (*C*). The same results were obtained when T cells from patient 1 and patient 3 were used (not shown).

$CD4^+$ subset, secretory lysosomes are not as well defined. The intracellular trafficking of CTLA-4 may also use similar but distinct pathways within these two subsets and may be more affected in the $CD8^+$ T cells when LYST is impaired. However, it is possible that the CD4 T cells are also affected, considering CTLA-4 surface expression during the course of the lymphoproliferative syndrome as observed in patient 1, but this hypothesis will be difficult to address because of the small number of patients available.

The reason for such a difference between mouse and human T lymphocytes is unknown, because it is the same gene that is affected. However, differences in the cytotoxic granule architecture and in the compartmental distribution of lytic enzymes have been described (39). The same kinds of difference between mouse and human have also been described in B cells. In B cell lines of CHS patients, intracellular trafficking of the MHC class II molecules is disturbed (14). These proteins are directed to an enlarged multilaminar vesicle compartment unable to undergo exocytosis, in which they accumulate. Such multilaminar compartments, which are responsible for the large structures in CHS B cells, are absent from mouse B lymphocytes (46), suggesting that there are structural differences in the abnormal granules of the two species.

The *beige* mouse is the murine homologue of human CHS. However, the lymphoproliferative syndrome has never been described in these mice. In contrast, almost all of the CHS patients evolved to the accelerated phase. We therefore propose that defective intracellular protein cycling, caused by *LYST* gene anomalies in CHS, impairs the expression of CTLA-4 and possibly of other regulatory molecules, disturbing the homeostasis of the immune system.

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