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# Human CTLA-4 Is Expressed *in Situ* on T Lymphocytes in Germinal Centers, in Cutaneous Graft-*versus*-Host Disease, and in Hodgkin's Disease

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**Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4, CD152) is a molecule expressed on *in vitro* activated T cells. CTLA-4 shares important sequence homology with CD28 and binds to the same ligands, CD80 (B7-1) and CD86 (B7-2). CTLA-4 probably functions as a negative regulator of T lymphocyte activation in the mouse, although this remains to be proven for human T lymphocytes. We have developed new monoclonal antibodies against human CTLA-4 and have investigated the *in situ* expression of CTLA-4 in a wide variety of normal and pathological human tissues expressing CD80 and CD86. As revealed in this study, CTLA-4 is expressed on thymocytes in thymic medulla, on a subset of $CD4^+$ T lymphocytes in germinal centers of follicular hyperplasia, on T cells, mainly $CD8^+$, infiltrating skin affected by graft-*versus*-host disease, and on T cells, mainly $CD4^+$, infiltrating Hodgkin's disease lesions. In immunoelectron microscopy, CTLA-4 was found on the plasma membrane as well as in the hyaloplasm and cytoplasmic vesicles, in agreement with its pattern of expression on *in vitro* activated T cells. Interestingly, no or at most scarce expression of CTLA-4 was found in granulomatous lymph nodes, T-cell-mediated inflammatory diseases, or non-Hodgkin's lymphomas, regardless of their expression of CD80 or CD86. Thus, expression of CTLA-4 appears to be induced in selective pathological conditions *in vivo*. The pathways leading to selective induction of CTLA-4 and its role in the pathophysiology of these conditions need to be further investigated.** ***(Am J Pathol 1998, 152:963–973)***

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4, CD 152) was originally discovered in a cDNA library derived from activated T cells. CTLA-4 is a single V-region member of the immunoglobin gene superfamily and shares extensive homology with CD28 at the nucleotide and the amino acid level. The genes for CD28 and CTLA-4 are located within close vicinity of one another, ie, on chromosome 1 band C in the mouse and on chromosome 2q33 in the human. The natural ligands for CTLA-4 are B7-1 (CD80) and B7-2 (CD86), membrane receptors that were originally identified as natural ligands for CD28 and that bind to CTLA-4 with a 10- to 50-fold higher affinity.[1–5]

Over the past several years, the CD28/CTLA-4/CD80/CD86 receptor system has emerged as a key control point in pathways leading to T cell activation.[3,6] CD28 functions as the receptor for a co-stimulatory signal leading to productive T cell activation and preventing the induction of anergy. Despite the many similarities with CD28, CTLA-4 probably fulfills a distinct role in T cell activation. Thus, CTLA-4-deficient mice develop a rampant and rapidly lethal T-lymphoproliferative disease with splenomegaly, lymphadenopathy, and multiorgan T-lymphocytic infiltration. T cells isolated from these mice proliferate spontaneously *in vitro*, as a result of unopposed or uncompeted co-stimulatory interactions between CD80/CD86 and CD28.[7–9] In addition, the proliferation, interleukin-2 production, and cell cycle progression of activated wild-type mouse T cells *in vitro*, are strongly inhibited by multivalent ligation of CTLA-4.[10–12] Finally, selective disruption of interactions of CTLA-4 with CD80 or CD86, eg, in the presence of anti-CTLA-4 Fab, promotes alloantigen-induced proliferation of wild-type lymphocytes *in vitro*,[13] whereas administration of anti-CTLA-4 *in vivo* exacerbated experimental allergic encephalomyelitis, promoted anti-tumor immunity, and accelerated development of the T cell immune response to nematode infection.[14–17] Based on these data, the prevailing opinion today is that CTLA-4, at least in the mouse, exerts a negative regulatory role in T cell activation, which is in sharp contrast with the pivotal co-stimulating role of CD28 in the initiation and progression of T cell immunity.[6,18]

In further contrast with CD28, which is constitutively expressed on resting T cells *in vitro*, CTLA-4 expression is

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largely, if not exclusively, restricted to activated T lymphocytes.[19–22] Little is known today about the expression of CTLA-4 in human tissues, except that synovial fluid T cells from patients with rheumatoid arthritis but not osteoarthritis or psoriatic arthritis express this molecule,[23] as do some intra- and interfollicular T lymphocytes in human tonsils.[24,25] In the mouse, expression of CTLA-4 has also been described on thymocytes.[26]

We have undertaken an immunohistochemical study on the *in situ* expression of CTLA-4 in human tissues, using newly developed anti-human CTLA-4 monoclonal antibodies (MAbs). Previously, CD80 and CD86, physiological ligands for CTLA-4, have been reported to be expressed on several cell types, the most important of which were 1) cells of the dendritic cell system, eg, Langerhans' cells in skin, interdigitating dendritic cells in T-cell-dependent zones of lymph nodes and spleen, veiled cells in lymph node sinuses, and fetal thymus dendritic cells located at the corticomedullary junction; 2) macrophages and epithelioid cells in granulomatous inflammations and antigen-presenting mononuclear cells in the gut mucosa of patients with inflammatory bowel disease; 3) a subset of germinal center B cells and B immunoblasts in lymph node and spleen; 4) T cells from rheumatoid synovial membranes; 5) Reed-Sternberg cells in Hodgkin's disease, and 5) neoplastic cells in anaplastic large-cell T-cell non-Hodgkin's lymphoma and follicular B-cell non-Hodgkin's lymphoma.[23,27–32] A selection of these tissues were analyzed for expression of CTLA-4.

## Materials and Methods

### Cells, Cell Lines, and Cell Culture

T lymphocytes from healthy adult volunteers were isolated by density centrifugation over Lymphoprep (density of 1.077 g/ml; Nycomed Pharma, Oslo, Norway), followed by antibody- and complement-dependent cell lysis with Lympho-KWIK-T (One Lambda, Los Angeles, CA) in combination with complement-fixing anti-CD16 (Leu-11b, Becton Dickinson, Erembodegem, Belgium) and anti-CD56 MAbs (NKH1A, Coulter, Hialeah, FL). This procedure yields populations containing more than 96% $CD3^+$ cells.[33] T lymphocytes were cultured in complete medium, RPMI 1640 (Gibco Life Sciences, Paisley, UK) supplemented with 1 mmol/L nonessential amino acids, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate (Gibco), 10 mg/L ofloxacin (Hoechst, Frankfurt am Main, Germany), and 10% fetal bovine serum (Gibco). A cell line secreting human CTLA-4-Ig protein was a gift from Dr. A. Lanzavecchia, Basel Institute of Immunology (Basel, Switzerland). Purified phytohemagglutinin was purchased from Wellcome Diagnostics (Charlotte, NC).

### Production of MAbs against huCTLA-4

Human (hu)CTLA-4 cDNA was cloned by reverse transcriptase polymerase chain reaction with huCTLA-4-specific primers from RNA extracted from purified and stim-

**Table 1.** Tissues Examined in This Study

| Tissue | Diagnosis | Number of cases |
|---|---|---|
| Lymph node, nonmalignant | Follicular hyperplasia | 20 |
| | Polymorphous pulp hyperplasia | 5 |
| | Dermatopathic lymphadenitis | 4 |
| | Cat scratch disease | 4 |
| | Sarcoidosis | 5 |
| | Tuberculosis | 4 |
| | Toxoplasmosis | 4 |
| Lymph node, Hodgkin's disease | Nodular sclerosis | 5 |
| | Mixed cellularity | 5 |
| | Lymphocyte predominance | 5 |
| Lymph node, Non-Hodgkin's lymphoma | B cell type | |
| | Small lymphocytic lymphoma | 3 |
| | Mantle cell lymphoma | 1 |
| | Follicular lymphoma | 10 |
| | Monocytoid B cell lymphoma | 1 |
| | Large-cell lymphoma | 3 |
| | Small non-cleaved lymphoma | 2 |
| | T cell type | |
| | Peripheral T cell lymphoma | 1 |
| | Anaplastic large cell lymphoma | 4 |
| | Mycosis fungoides | 1 |
| Thymus Skin | Normal adult thymus | 3 |
| | Acute graft-*versus*-host disease | 3 |
| | Chronic graft-*versus*-host disease | 2 |
| | Psoriasis | 3 |
| | Toxic dermatitis | 1 |
| | Contact dermatitis | 4 |
| | Dermatomyositis | 1 |
| Colon | Ulcerative colitis | 3 |
| | Crohn's disease | 4 |

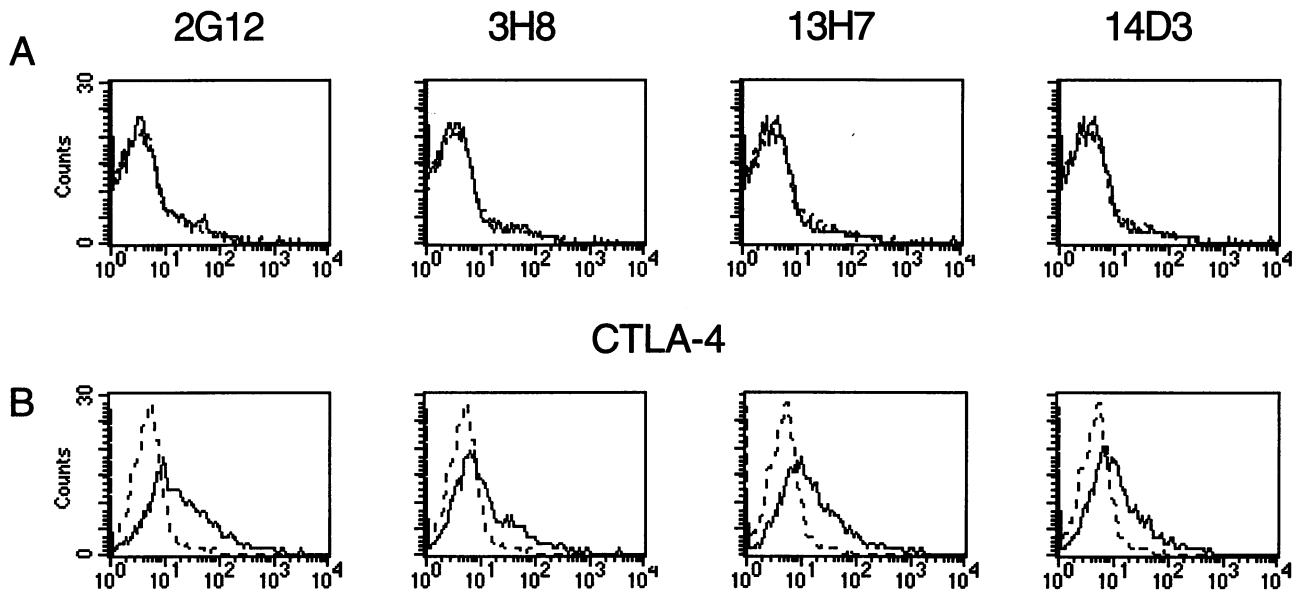


**Figure 1.** Characterization of anti-CTLA-4 MAbs. T cells were stained with anti-CTLA-4 MAb as indicated (—) or with mouse IgG control (- - -), followed by biotinylated goat anti-mouse Ab and streptavidin-PE. **A:** Resting T cells. Histograms of control Ig or anti-CTLA-4 staining of resting T cells essentially overlap, demonstrating that none of the MAbs bind to resting T cells. **B:** Purified T cells after 72 hours of culture with 1 $\mu$g/ml phytohemagglutinin.

ulated human T cells. After insertion in the baculoviral transfer vector, recombinant baculoviruses were generated following the baculogold transfection approach, according to the manufacturer's instructions (PharMingen, San Diego, CA). Balb/C mice were immunized with $5 \times 10^6$ huCTLA-4-transformed Sf9 cells at 0, 1, and 3 months. After the day 90 boost, animals were sacrificed and splenocytes were fused with the nonsecreting Sp2/0 myeloma line (American Type Culture Collection, Rockville, MD) using standard procedures and selected in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L nonessential amino acids (Gibco), 10 mg/L ofloxacin (Hoechst), 0.1 mmol/L hypoxanthine, 0.01 mmol/L aminopterin, and 0.016 mmol/L thymidine (Boehringer Mannheim, Mannheim, Germany). In this study, the following anti-CTLA-4 MAbs were used: 2G12 (IgG2a), 3H8 (IgG3), 13H7 (IgG3), and 14D3 (IgG2a).

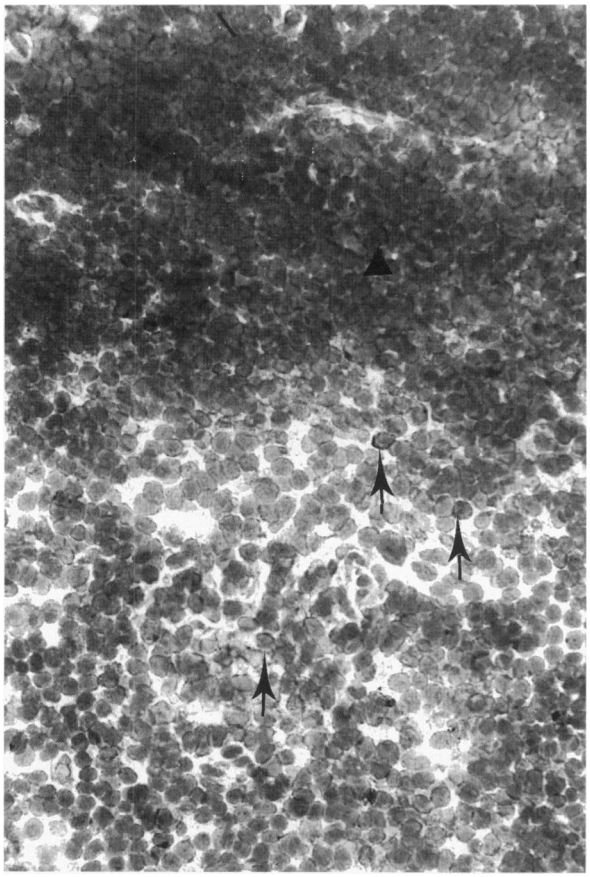

**Figure 2.** Normal adult thymus. Most CTLA-4-positive T lymphocytes are present in the thymic medulla (**arrow**); only few are located in the cortex (**arrowhead**). Magnification, ×300.

## *Flow Cytometry*

Cells were suspended in 100 $\mu$l of PBS with 0.1% $NaN_3$, supplemented with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The cell suspension was incubated with primary MAb for 30 minutes on ice, followed by two washes, stained with secondary reagents and fixed with Cellfix (Becton Dickinson). Phycoerytrin (PE)-conjugated Leu-28 (anti-CD28), fluorescein isothiocyanate (FITC)-conjugated Leu-1 (anti-CD5) and streptavidin-PE were purchased from Becton Dickinson. Biotinylated goat anti-mouse $F(ab')_2$ IgG was obtained from Immunotech (Marseille, France) and mouse IgG from Jackson ImmunoResearch Laboratories (West Grove, PA). Immunostaining was measured on a FACScan flow cytometer using CellQuest software (Becton Dickinson), with electronic gates set in forward and side scatter, and/or CD5 positivity.

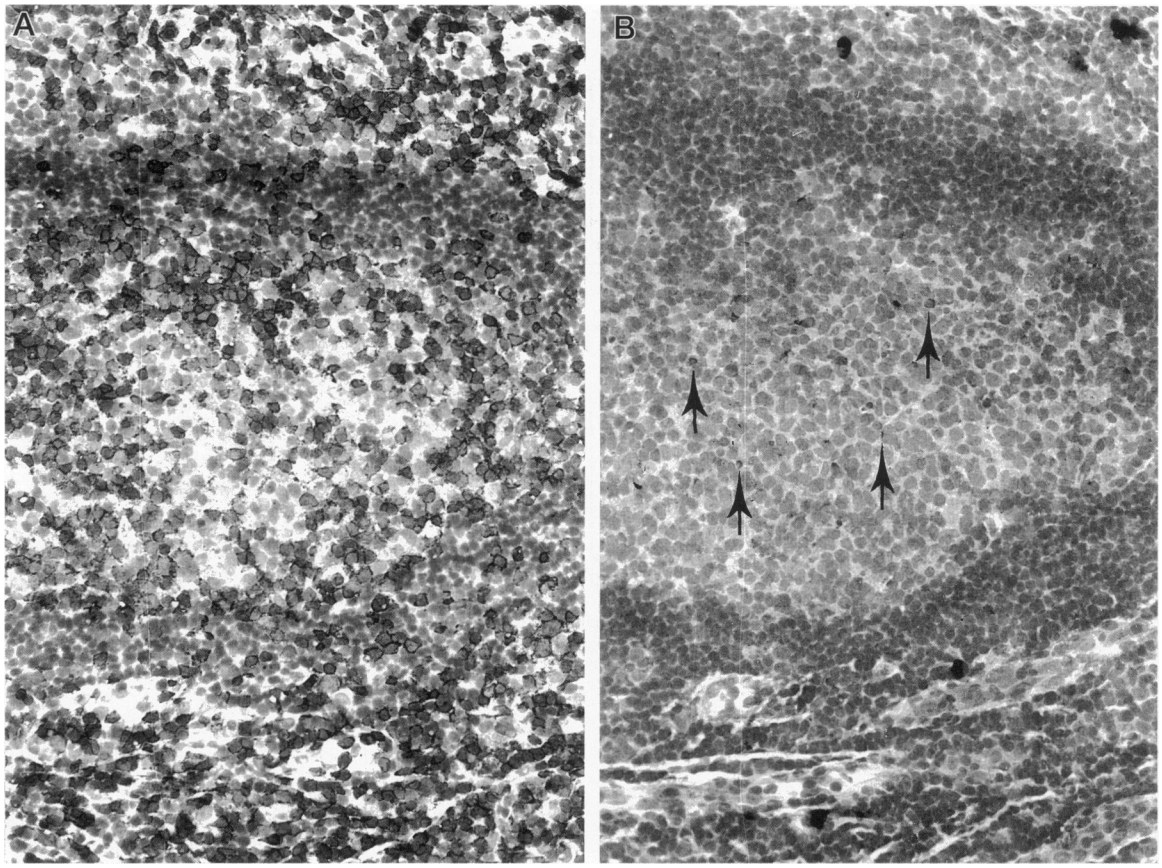


**Figure 3.** Follicular hyperplasia. **A:** Secondary lymph follicle showing numerous CD3-positive T lymphocytes in the germinal center as well as in the mantle. **B:** Only a subset of these lymphocytes express CTLA-4 (**arrow**). Magnification, ×190.

### *Immunohistochemistry*

A list of tissues, analyzed for the expression of CTLA-4, is given in Table 1. All tissue samples had been obtained for diagnostic purposes. Part of these samples had been preserved for research purposes, were snap-frozen in liquid nitrogen-cooled isopentane, and stored at −70°C until used for immunohistochemistry. Acetone-fixed cryostat sections (4 μm) were used. The samples were incubated with a mixture of MAbs 2G12, 3H8, 13H7, and 14D3, in a final concentration of 5 μg/ml each, followed by biotinylated rabbit anti-mouse Ab and subsequently by peroxidase-conjugated ABC complex (Dakopatts, Glostrup, Denmark).[29] Aminoethylcarbazole and $H_2O_2$ were used as peroxidase substrates. Controls consisted of replacement of the primary antibodies by irrelevant MAbs of identical isotype.

For double staining, an immunofluorescence technique was used. Tissue sections were incubated with the mixture of 2G12, 3H8, 13H7, and 14D3, followed by a biotinylated rabbit anti-mouse Ab and developed with streptavidin-PE. As a last step, sections were counterstained with FITC-conjugated Leu-4 (anti-CD3), FITC-conjugated Leu-3a (anti-CD4), or FITC-conjugated Leu-2a (anti-CD8; Becton Dickinson).

For immunoelectron microscopy, small tissue samples were fixed and stained as previously described.[34] Briefly, samples were fixed in a mixture of 0.25% glutaraldehyde and subsequently fixed in 4% paraformaldehyde. After borohydride treatment and cryoprotection, small specimens were frozen in liquid freon cooled by liquid nitrogen and stored at −80°C until further use. For immunostaining, the frozen tissue samples were thawed in phosphate buffer. The 20- to 40-μm sections were cut with a tissue chopper and stained with an indirect immunoperoxidase procedure using the mixture of 2G12, 3H8, 13H7, and 14D3. The stained sections were post-fixed in $OsO_4$, dehydrated, and embedded in Epon. Ultra-thin sections were cut and examined in the electron microscope with or without counterstaining with uranyl acetate and lead citrate. For control, the primary or secondary antibody was omitted.

## *Results*

### *Characterization of Anti-CTLA-4 MAbs*

For the purpose of this study, a mixture of four different anti-CTLA-4 MAbs, ie, 2G12 (IgG2a), 3H8 (IgG3), 13H7 (IgG3), and 14D3 (IgG2a), was used. Hybridoma supernatants had been screened for the presence of anti-CTLA-4 MAb in an ELISA measuring the reactivity of their supernatants with human CTLA-4-Ig but not with polyclonal human Ig. After limiting dilution cloning, the secretion of anti-CTLA-4 MAb by these hybridomas was confirmed in flow cytometry by demonstrating the specific

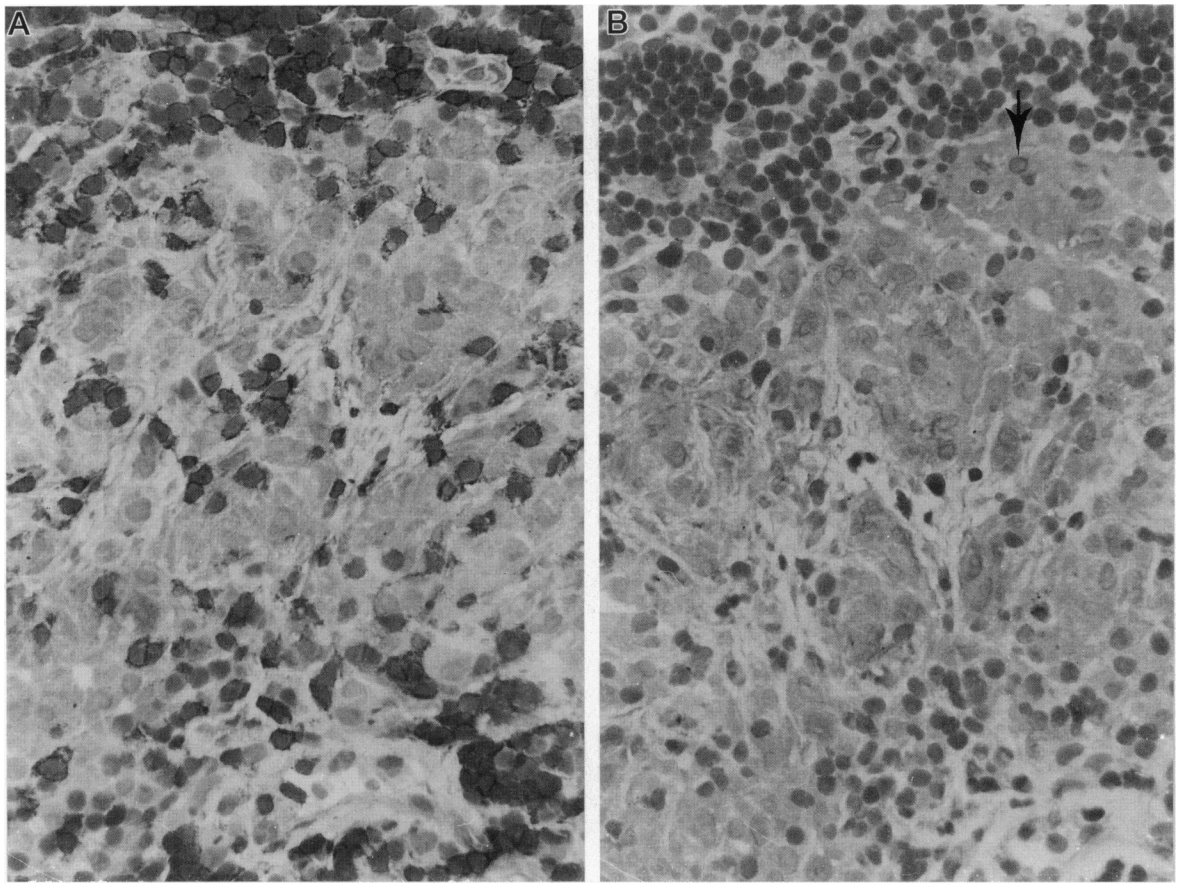


**Figure 4.** Sarcoid granuloma. Numerous CD3-expressing T cells (**A**) but virtually no CTLA-4-expressing cells (**B, arrow**) can be seen. Magnification, ×300.

reactivity of their supernatants to 1) purified human T lymphocytes after 3 days of activation *in vitro* with phytohemagglutinin at 1 μg/ml but not to resting T cells (Figure 1, A and B) and 2) Sf9 insect cells transfected with huCTLA-4 but not with sham-transfected cells (not shown). Preincubation of purified resting T cells with the anti-CTLA-4 MAbs did not diminish subsequent staining with anti-CD28 MAb, ruling out cross-reactivity of these MAbs with CD28 (not shown).

## *Thymus*

In thymic tissue, obtained from adults undergoing lung surgery, a limited number of thymocytes, mainly located in the medulla, was positive for CTLA-4 (Figure 2).

## *Inflammatory Lymphoid Tissues*

Lymph nodes diagnostic of various inflammatory conditions were chosen based on the characteristic hyperplasia of the various functional compartments to allow easier identification of antibody-reactive cells. In all cases of follicular hyperplasia, a subset of lymphocytes within the germinal center and, to a lesser extent, in the follicle mantle were reactive with anti-CTLA-4 (Figure 3, A and B). The majority of positive cells appeared to be located within the dark zone of the follicle center, ie, where the centrocytes are located. Double-immunofluorescence studies revealed that anti-CTLA-4-reactive cells in the germinal center were uniformly CD3 positive (not shown) and CD4 positive (see Figure 8, A and B). By semiquantitative estimation, approximately 10% of T cells in the germinal center expressed CTLA-4.

Dermatopathic lymphadenitis is a condition typically seen in patients with a generalized itching dermatosis. In these lymph nodes, the T cell areas are particularly well developed, with abundant expression of CD80 on interdigitating dendritic cells in the paracortex and on veiled cells in the afferent lymph node sinuses.[29] However, contrary to our expectation, only a low number of scattered T lymphocytes in the paracortex were reactive with anti-CTLA-4 in these areas. Also, in cases of polymorphous pulp hyperplasia, where CD80-positive B immunoblasts are present, only rare CTLA-4-positive T lymphocytes were found. The same was true in various forms of granulomatous inflammation, including sarcoidosis, cat scratch disease, tuberculosis, and toxoplasmosis (Figure 4).

## *Inflammatory Nonlymphoid Tissues*

We also evaluated the expression of CTLA-4 in a number of nonlymphoid tissues affected by established or putative T-cell-mediated inflammatory pathology. No signifi-

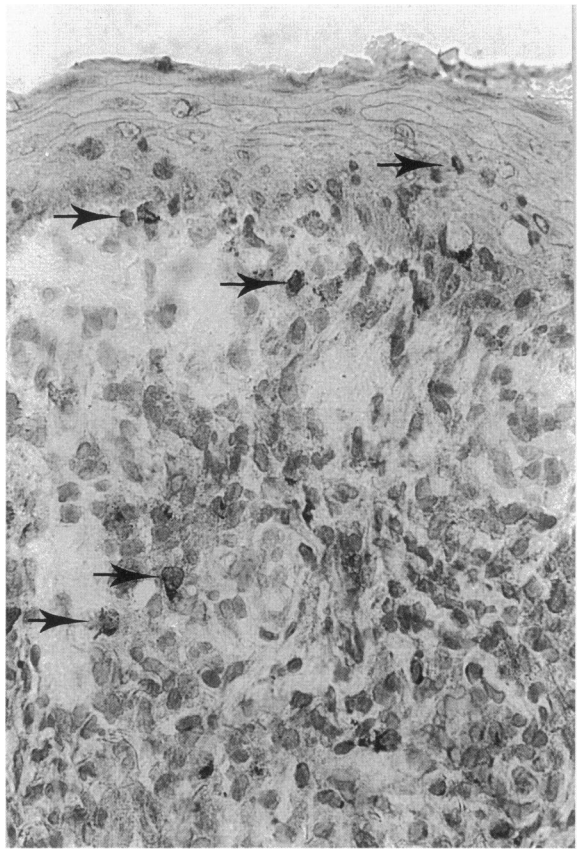

**Figure 5.** Acute cutaneous graft-*versus*-host disease. Numerous CTLA-4-expressing T lymphocytes are present at the dermo-epidermal junction as well as in the dermis (**arrow**). Few CTLA-4-positive cells are present in the epidermis as well (**arrow**). Magnification, ×300.

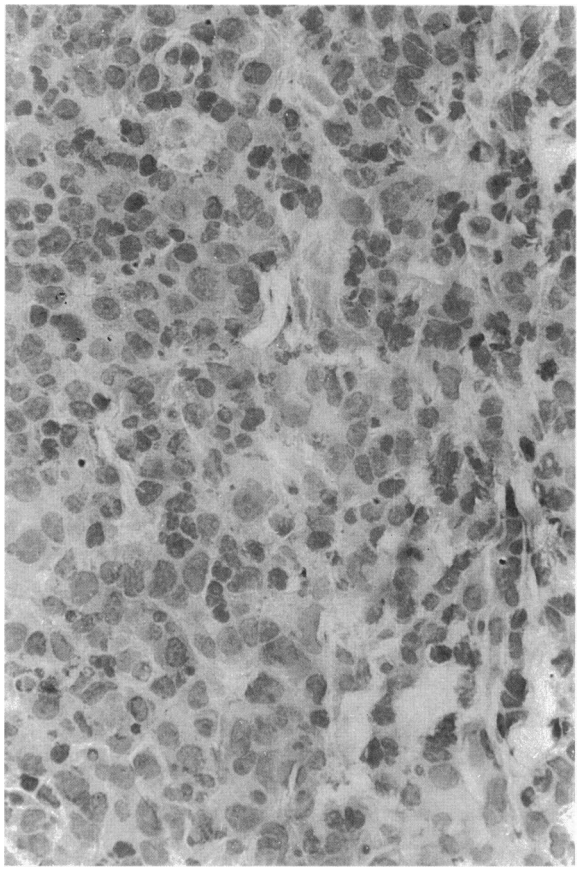

**Figure 6.** Anaplastic large-cell lymphoma. Virtually no infiltrating CTLA-4-positive T lymphocytes can be seen. Magnification, ×300.

cant numbers of cells reactive with anti-CTLA-4 were found in skin biopsies of patients suffering from psoriasis, dermatomyositis, toxic dermatitis, or contact dermatitis, despite the presence of an obvious T-lymphocytic infiltrate. In contrast, infiltrating lymphocytes in the superficial dermis and the epidermis were found strongly reactive with anti-CTLA-4 MAb in skin biopsies from patients suffering from acute (Figure 5A) or chronic graft-*versus*-host disease (not shown). Detailed patient characteristics are listed in Table 2. In two of the three patients with acute graft-*versus*-host disease, acute graft-*versus*-host disease was a consequence of donor lymphocyte infusions for disease relapse after allogeneic matched sibling transplantation. In the third patient, acute graft-*versus*-host disease occurred as a complication of liver transplantation. In the latter case, the diagnosis was established by the demonstration of mixed chimerism of white blood cells in peripheral blood, by means of DNA typing of HLA class II antigens.[35] CTLA-4 could also be detected in skin biopsies taken in patients with clinical chronic graft-*versus*-host disease (not shown). Expres-

**Table 2.** Clinical Characteristics of Patients with Graft-*versus*-Host Disease

| Patient | Sex (M/F) | Age (years) | Primary disease | Type of transplantation | DLI | Clinical diagnosis | Clinical symptoms | Onset | Time of biopsy |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 46 | MDS | MSD | d 160 | Acute GVHD, grade II* | Skin rash >50%, fever, nausea, diarrhea >500 ml/d | d 172 | d 181 |
| 2 | M | 39 | MDS | MSD | d 207 | Acute GVHD, grade III* | Generalized erythroderma, fever, pancytopenia, disturbed liver function | d 230 | d 231 |
| 3 | M | 65 | Liver cirrhosis | liver Tx | | Acute GVHD, grade III* | Generalized erythroderma, fever, pancytopenia | d 23 | d 34 |
| 4 | M | 42 | AML | MSD | | Chronic GVHD, extensive, progressive onset | Generalized hyperpigmentation, nausea, diarrhea, disturbed liver function | d 38 | d 66 |
| 5 | F | 17 | AML | MSD | | Chronic GVHD, extensive | Oral ulcers, sclerodermoid skin lesions, hyperpigmentation | d 138 | d 195 |

Donor lymphocyte infusions (DLI) were administered to patients #1 and 2 because of disease relapse on days 136 and 180, respectively. M, male; F, female; MDS, myelodysplastic syndrome; AML, acute myelogenous leukemia; MSD, matched sibling marrow donor; Tx, transplantation (date of transplantation is referred to as day 0); d, day; GVHD, graft-*versus*-host disease.

*Grading according to Przepiorka et al.[42]

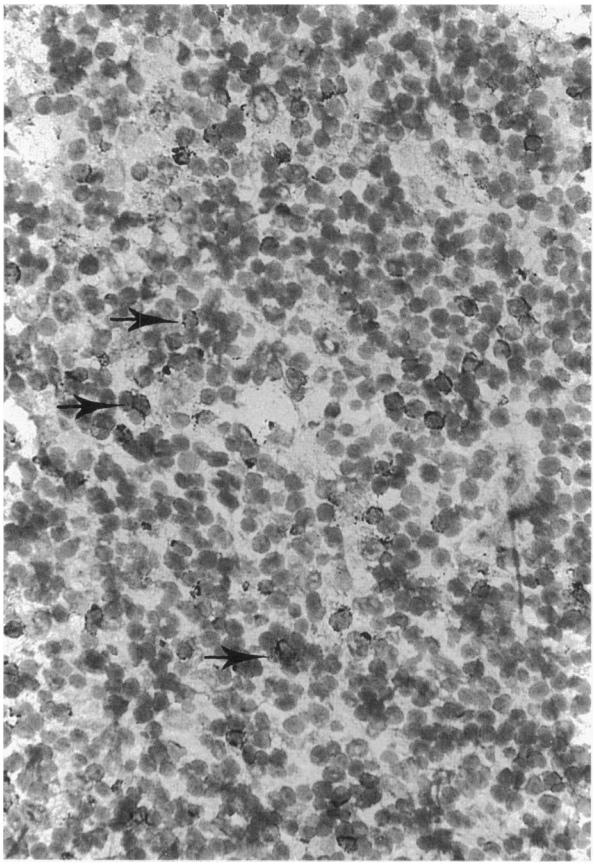

Figure 7. Nodular sclerosis Hodgkin's disease. A high number of CTLA-4-expressing T lymphocytes are present in the tissue (arrow). Most of these cells do not immediately surround Reed-Sternberg cells. Magnification, ×300.

sion of CTLA-4 on T cells was not detectable by flow cytometry on peripheral blood T lymphocytes obtained in 11 patients at various times after allogeneic transplantation (not shown). Therefore, CTLA-4 expression does not appear to be a nonspecific consequence of the transfer of allogeneic T cells or of reconstitution of the T cell repertoire after allogeneic bone marrow transplantation. In double-immunofluorescence studies, CTLA-4 was predominantly expressed on CD8-positive T cells (see Figure 8, C and D).

In bowel lesions of ulcerative colitis and Crohn's disease, which are also featured by a significant T-lymphocytic infiltration, no anti-CTLA-4-reactive cells were found outside the germinal centers.

### *Malignant Lymphomas*

CTLA-4 appears to be a T-cell-specific marker in T cell activation assays *in vitro* and by immunohistochemistry performed on non-neoplastic tissues. Biopsies obtained from various subtypes of T-cell non-Hodgkin's lymphomas were analyzed for the expression of CTLA-4 (Table 1). However, none of these lymphomas were reactive with anti-CTLA-4. No CTLA-4 positivity could be found on T cells infiltrating $CD80^+$ or $CD86^+$ non-Hodgkin's lymphoma, such as follicular B-cell non-Hodgkin's lymphoma or anaplastic large-cell non-Hodgkin's lymphoma (Figure 6). However, in Hodgkin's disease, T cells within and outside the T-rosettes exhibited a strong reactivity with CTLA-4. The number of T cells reactive with anti-CTLA-4 was most marked in the nodular sclerosing subtype (Figure 7). Anti-CTLA-4 reactivity was mainly found on CD4-positive cells in double immunofluorescence, although some CTLA-4-positive cells were CD8 positive (not shown).

### *Immunoelectron Microscopy*

Although having structural characteristics of plasma-membrane-anchored proteins, CTLA-4 is predominantly localized in an intracellular Golgi or post-Golgi compartment within activated T cells *in vitro*. From this compartment, it can become focally externalized to membrane areas of interaction with anti-cell receptor MAbs or with antigen-presenting cells.[36,37] We studied the subcellular distribution of CTLA-4 *in situ* in hyperplastic tonsillar tissue by a combination of immunohistochemistry and electron microscopy. Both the intensity and the localization of anti-CTLA-4 reactivity varied between the T lymphocytes examined. Consistent with previously published *in vitro* data,[36,37] CTLA-4 could be found in the cytoplasm of positive cells (either in the hyaloplasm or some cytoplasmic vesicles), on the membrane, or on both (Figure 9).

## *Discussion*

In the present study on the expression of human CTLA-4 *in situ*, CTLA-4 was found on CD4-positive T cells within germinal centers of follicular hyperplasia, on T cells (predominantly CD8 positive) in cutaneous graft-*versus*-host disease, and on infiltrating T cells (predominantly CD4 positive) in Hodgkin's disease.

In lymphoid tissues, CTLA-4 expression was consistently detected on a subset of CD4-positive T cells within the germinal center of follicular hyperplasia, as previously reported.[24,25] These cells were preferentially localized in the dark zone of the germinal center, where the centrocytes are situated. T cells within the germinal center are known as a specific phenotypic population expressing CD4, CD45RO, and CD57 but not CD62L. They are known to participate in complex bidirectional interactions with centroblasts and centrocytes that are driven by antigen and that involve various adhesive and co-stimulating receptor/counter-receptor systems: LFA-1, LFA-3, CD40, and CD80/CD86 on B lymphocytes and CD54, CD2, CD40L, and CD28/CTLA-4 on the T cell. Driven by these multiple interactions, hypermutation of the IgV region takes place in centroblasts residing in the light zone, followed by positive selection and maturation to the smaller centrocytes, which are located in the dark zone. The latter will ultimately leave the germinal center and give rise to antibody-producing plasma cells or memory B cells.[38–40] The predilection for CTLA-4-positive T cells to be located in the dark zone of the germinal center may suggest that CTLA-4 is directly or indirectly involved in feedback mechanisms controlling the expansion of ger-

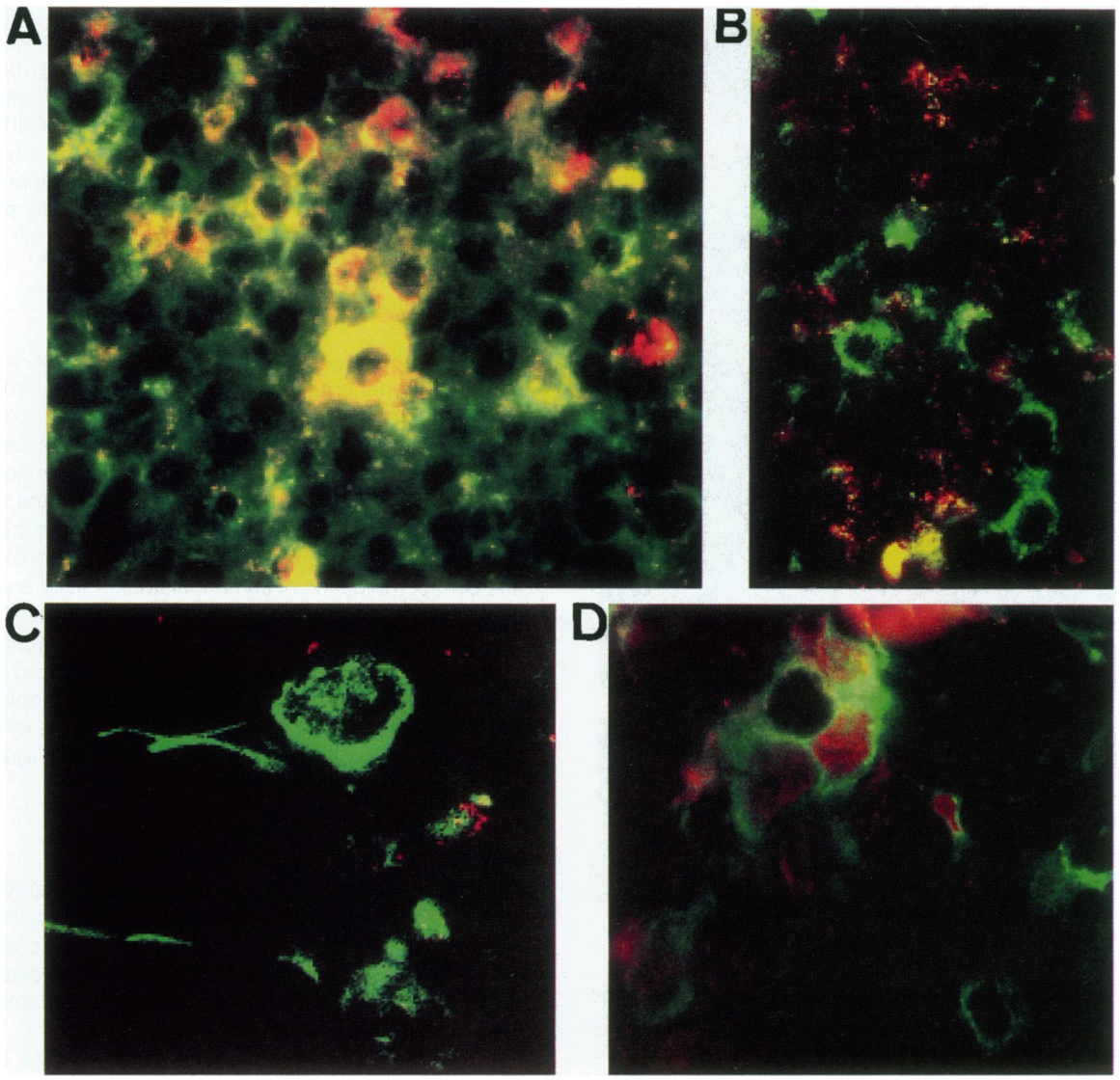


**Figure 8.** Two-color immunofluorescence of anti-CTLA-4 MAb and anti-CD4 or anti-CD8 MAb. Sections were stained with anti-CTLA-4 MAb, followed by a biotinylated rabbit anti-mouse Ab and streptavidin-PE (red) and with anti-CD4-FITC (green, **A** to **C**) or anti-CD8-FITC (green, **B** to **D**). **A** and **B:** Follicular hyperplasia. CTLA-4-positive cells react with anti-CD4-FITC but not with anti-CD8. Magnification, ×630. **C** and **D:** Acute graft-*versus*-host disease. The majority of CTLA-4-positive cells express CD8 but not CD4. Magnification, ×1000.

minal centers or the transition to memory B cells or antibody-producing plasma cells. CTLA-4 can reportedly also be induced on B cells *in vitro* when cultured in the presence of membranes from activated T cells that express the ligand for CD40.[21] *In situ*, however, no evidence was found for expression of CTLA-4 on germinal center B cells.

Bright expression of CTLA-4 was found on a majority of infiltrating $CD8^+$ T lymphocytes in skin biopsies of graft-*versus*-host disease. In the cases examined, acute graft-*versus*-host disease was a consequence of liver transplantation or of donor lymphocyte infusions for relapse after allogeneic bone marrow transplantation. In this context, other causes of cutaneous eruptions, such as acral erythema, radiation-induced dermatitis, and lymphocyte recovery, that occur in the early interval after allogeneic bone marrow transplantation, can readily be ruled out. In addition, in none of the three patients was there evidence supporting viral infections, and in the bone marrow transplantation patients, no recent additions had been made to their prescriptions. No CTLA-4 could be detected on peripheral blood T lymphocytes obtained from 11 patients at various time points after allogeneic transplantation (not shown). Therefore, the CTLA-4 expression of the T lymphocytic infiltrate in cutaneous graft-*versus*-host disease probably reflects the activated state of a selected population of T cells participating in the development of graft-*versus*-host disease. Molecular studies of the T cell repertoire in cutaneous lesions of graft-*versus*-host disease have indeed indicated that the T-lymphocytic infiltrate in graft-*versus*-host disease constitutes a selected population of donor T lymphocytes.[41] CTLA-4 expression was not observed in a variety of other inflammatory skin lesions that are mediated by T cells, outside the setting of allogeneic transplantation. It would be interesting to know whether, in the early period after allogeneic bone marrow

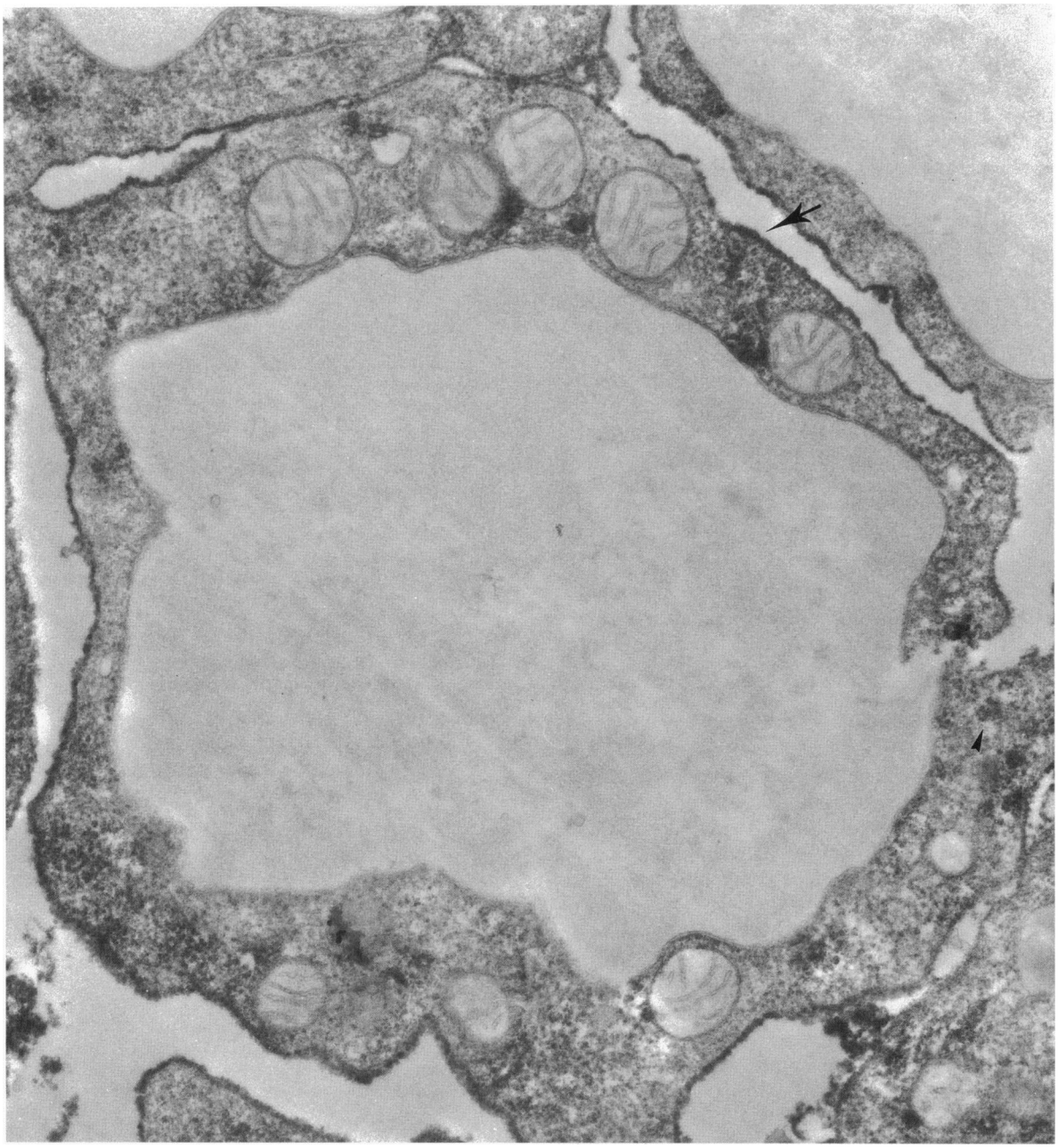

**Figure 9.** Subcellular localization of CTLA-4. Electron micrographs detecting CTLA-4 in lymphocytes of hyperplastic lymph node and of hyperplastic tonsil show peripheral membrane (**arrow**) and cytoplasmic (**arrowhead**) localization of CTLA-4. Indirect immunoperoxidase without counterstaining; magnification, ×28,750.

transplantation, CTLA-4 expression on the lymphocytic infiltrate would allow us to distinguish between radiation-induced dermatitis, viral exanthems, drug eruptions, cutaneous eruption of lymphocyte recovery, and cutaneous graft-*versus*-host disease, a differential diagnosis that is notoriously difficult. However, as the policy in our center is not to take biopsies during this time interval and to rely on clinical judgment for treatment decisions, we do not have the material to carry out this study at the present time.

An interesting finding of this study was the bright expression of CTLA-4 on human T cells in Hodgkin's disease. Reed-Sternberg cells express CD80 and CD86, molecules that have been shown to contribute to the allostimulatory capacity of these cells.[27,28,30,32] Most of the non-Hodgkin's lymphomas, in contrast, do not express CD80 or CD86. Therefore, it is not entirely unexpected that no CTLA-4 was found on tumor-infiltrating lymphocytes in these cases. However, in anaplastic large-cell lymphoma, which does express CD80/CD86, and in follicular lymphoma, which expresses CD80/CD86 and CD40, no significant numbers of CTLA-4-positive lymphocytes were found either. In the case of anaplastic large-cell lymphoma, the predominant presence of tumor cells in the lymph node sinuses but not in the parenchyma, where most of the interactions with T cells occur, could account for this. An alternative possibility would be that Reed-Sternberg cells have a peculiar capacity to

induce CTLA-4 on T cells by mechanisms other than the expression of known co-stimulatory molecules. However, we performed primary or secondary mixed lymphocyte reactions of peripheral blood mononuclear cells from healthy volunteers against the Hodgkin's disease-derived cell line KM-H2 (which is $CD80^+$ and $CD86^+$). In these cultures, we could not find higher levels of CTLA-4 on T cells compared with mixed lymphocyte reactions against other $CD80^+/CD86^+$ Epstein-Barr virus-transformed B cell lines (unpublished results). Finally, the regulation of CTLA-4 expression on T cells in patients suffering from Hodgkin's disease might be aberrant. Assuming that CTLA-4 negatively regulates T cell activation, it could be speculated that Reed-Sternberg cells escape from anti-tumor immunity by inducing and interacting with CTLA-4 on T lymphocytes.[15] One may also wonder about the relation between the expression of CTLA-4 and the marked deficit of T cell immunity in Hodgkin's disease. *In vitro* studies on T cells and tumor cells isolated from Hodgkin's disease biopsies and on peripheral blood T cells from patients with this disease will be required to further clarify these issues.

*In vitro* CTLA-4 behaves as a low-abundancy antigen with a transient expression after T cell activation. This can in part explain the limited reactivity of T cells with anti-CTLA-4 MAb in various other inflammatory conditions involving the T-cell compartment or the lymph node pulp. Similarly, only a few CTLA-4-expressing T lymphocytes were observed in various conditions characterized by granulomatous inflammation such as cat scratch disease, toxoplasmosis, tuberculosis, sarcoidosis, and Crohn's disease, despite the high number of CD80/CD86-expressing cells observed in these conditions. As was the case for the lymphomas, it is again unexplained why CTLA-4 is clearly found on T cells in certain conditions, eg, in cutaneous graft-*versus*-host disease or germinal centers of follicular hyperplasia, but not in a variety of other inflammatory conditions despite their expression of co-stimulatory molecules.

It is concluded that CTLA-4 is prominently expressed on T lymphocytes in cutaneous graft-*versus*-host disease, the thymic medulla, lymph node follicular hyperplasia, and Hodgkin's disease. Before CTLA-4 can be targeted in immune intervention, the pathways leading to CTLA-4 expression and the functional role of CTLA-4 in these conditions need to be further explored *in vitro*.

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