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CD30 Ligand Expression in Nonmalignant and Hodgkin's Disease-Involved Lymphoid Tissues

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The CD30 ligand (CD30L) is a type II transmembrane glycoprotein of the tumor necrosis factor ligand superfamily. Recent cloning of CD30L has enabled studies to explore its function and tissue distribution. For instance, recombinant CD30L has been shown to co-stimulate T cells and to act as mitogen for Hodgkin's disease (HD)-derived cell lines. The counter-receptor for CD30L, ie, CD30, is a type I cytokine receptor that is highly expressed by activated T cells, Hodgkin and Reed-Sternberg (H-RS) cells, and anaplastic large cell lymphoma cells. In the present study, recombinant membrane-bound and soluble human CD30L were instrumental to raise a monoclonal antibody (M80) recognizing membrane-bound CD30L on transfected and native cells. With this reagent, a panel of cultured lymphoma-derived cell lines as well as primary normal, reactive, and HD-involved lymphoid tissues were examined for expression of CD30L by immunostaining and flow cytometry. In reactive lymph nodes and tonsils, CD30L was expressed by a small subset of lymphoid cells, histiocytes, and granulocytes. Higher levels of CD30L expression were noted in HD lesions among bystander cells, ie, T cells and granulocytes that surrounded H-RS cells. Native CD30L displayed at the cell surface was functionally active as shown by the ability of fixed granulocytes to interact with CD30$^+$ cell lines. Moreover, CD30L was detectable, although to a lower staining intensity, in primary H-RS cells of all HD tissues investigated regardless of the histological subtype and the phenotype of H-RS cells (ie, CD30$^+$/CD40$^+$ versus CD30$^-$/CD40$^+$). Co-expression of CD30 and CD30L that was seen on H-RS cells of all, except the CD30$^-$ nodular lymphocyte predominant, subtypes of HD may point to the use of this pair of molecules in paracrine and/or autocrine mitogenic cell interactions. Monoclonal antibody M80 may thus represent a useful tool for studying CD30L expression on cultured cell lines and primary cells from normal, reactive, and malignant tissues. (Am J Pathol 1996, 149:469–481)

Hodgkin's disease (HD) is characterized by strong expression of the CD30 antigen mainly by Hodgkin and Reed-Sternberg (H-RS) cells.[1–3] CD30 has also been identified on activated or virally transformed B and T cells and a small subset of lymphoid cells in reactive tonsils and lymph nodes.[2–6] CD30 has been identified as a member of the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily.[7] By

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sequence homology in the extracellular domain with characteristic cysteine-rich repeats, this receptor family includes the low affinity nerve growth factor receptor (p75), two distinct receptors for TNF (TNF-RI, p60; TNF-RII, p80), TNF-receptor-related protein, CD27, CD30, CD40, 4–1BB, OX40, CD95 (FAS/APO-1), and several viral open reading frames encoding soluble homologues (reviewed in Refs. 8 and 9). Counterstructures/ligands have been identified for all TNF receptor superfamily members with TNF, lymphotoxin (LT)-α, LT-β, CD27L, CD30L, CD40L, 4–1BBL, OX40L, and CD95L (FASL) forming, in parallel, the existing TNF ligand superfamily (reviewed in Refs. 8 and 10).

We have recently reported on the molecular identification and characterization of a membrane-bound ligand for the CD30 receptor expressed by activated T cells.[11] Based on mRNA and surface binding studies using the CD30-Fc fusion protein, CD30L expression was found not only by activated T cells but also by stimulated monocytes/macrophages, granulocytes, subpopulations of B cells, and some Burkitt-like lymphoma cell lines.[11–14] Recombinant CD30L conferred proliferative effects to some HD-derived cell lines while exerting growth inhibition on $CD30^+$ anaplastic large cell lymphoma cells.[11,12] In addition, CD30L enhanced cytokine release from cultured H-RS cells and anaplastic large cell lymphoma cells, including interleukin (IL)-6, TNF, and LT-α, as well as from co-stimulated T cells, including IL-2, TNF, interferon (IFN)-γ.[15,16] Similarly, CD30L was shown to up-regulate surface expression of activation/adhesion molecules, such as CD54, CD80, and CD86, on the same $CD30^+$ cell types.[14–16]

Several lines of evidence indicate that cell growth and proliferation in HD is tightly regulated by a complex network of cytokine-mediated and cell-contact-dependent interactions among H-RS cells and hyper-reactive bystander cells (mainly T cells, histiocytes, and neutrophils) accumulating in HD-involved tissues.[17,18] Functional studies have suggested that CD30L may be one of the key molecules involved in the deregulated cellular network underlying HD.[14] Therefore, we have examined CD30L expression *in situ* to clarify its role in HD in greater detail. To this end, a monoclonal antibody (MAb) M80 was raised against recombinant human membrane-bound and soluble CD30L. By flow cytometry and immunocytochemistry, M80 was shown to specifically react with recombinant or native membrane-anchored CD30L. In reactive, nonmalignant lymphoid tissues, a small number of lymphoid cells was stained with M80 by immunocytochemistry. Also, some histiocytes and granulocytes appeared to express the CD30L. In single-cell suspensions of HD-involved tissues, CD30L expression was detected on the majority of H-RS cells in all HD specimens investigated, regardless of the histological subtype. Also, reactive bystander cells surrounding H-RS cells strongly displayed CD30L. The magnitude of CD30L expression was, however, much higher in reactive bystander cells present in HD-involved tissues than in the same cell populations seen in reactive lymphoid cells of nonmalignant lymphoid tissues. Taken together, our data suggest that CD30L may act as a paracrine and/or autocrine growth and activation factor and that the CD30L may play a pathobiological role in HD.

Materials and Methods

Cells and Reagents

Peripheral blood T cells were isolated from consenting healthy blood donors by centrifugation over Histopaque (Sigma Chemical Co., St Louis, MO) and rosetting with 2-aminoethylisothiouronium-bromide-treated sheep erythrocytes as previously described.[19] Preparations obtained in this way were always >94% $CD3^+$ by flow cytometry. These cells were subsequently activated with 50 ng/ml phorbol-12-myristate 13-acetate and 500 ng/ml ionomycin (Sigma) for 18 hours. Granulocytes were isolated from freshly drawn peripheral blood by adding 4.5% dextran (Sigma) in phosphate-buffered saline (PBS) to a final concentration of 2.25%. After sedimentation of erythrocytes for 30 minutes, the leukocyte-rich supernatant was recovered, washed, and centrifuged on a Percoll (Pharmacia, Uppsala, Sweden) cushion with an osmolality of 285 to 294 mOsmol/kg.[20] Respective interfaces were collected, and more than 95% pure granulocyte preparations (fraction 3) were stained according to Pappenheim or May-Grünwald-Giemsa for morphological evaluation and were utilized for surface staining or functional assays. The EBV^- Burkitt lymphoma cell line DG-75 (DSM-ACC 83), the HD-derived cell lines HDLM-2 and L-428 (DSM-ACC 17 and 197), and the $CD30^+$ anaplastic large cell lymphoma cell line KARPAS 299 (DSM-ACC 31) were kindly provided by Dr. H. G. Drexler, German Collection of Microorganisms and Cell Cultures/DSM (Braunschweig, Germany) and have been extensively described before.[12] Cells were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (Intergen Corp., Purchase, NY), 100 μg/ml penicillin/streptomycin, and 2 mmol/L L-glutamine

(Sigma) at 37°C in a humidified atmosphere of 7% CO_2 in air.

CV-1/EBNA cells[21] were transfected using the diethyl aminoethyl/dextran method with either vector alone (CV-1/HAV) or the human full-length CD30L-cDNA-containing expression vector (CV-1/CD30L) and were either fixed at day 2 post-transfection with 1% paraformaldehyde for 5 minutes at 25°C or were used for cytospin preparations.[11] Expression levels were measured by flow cytometry using the CD30-Fc fusion protein (soluble extracellular CD30 fused to the Fc region of human IgG_1) as described.[11] Constructs for a soluble form of CD30L (sCD30L-Fc) and CD27L (sCD27L-Fc) were linked at the amino terminus to the carboxyl-terminal end of the Fc domain of human IgG_1. The human sCD30L and sCD27L proteins were expressed in CV-1/EBNA cells and purified using protein A columns.

To generate antibodies against the human CD30L, CB6F1 mice (purchased from Jackson Laboratories, Bar Harbor, ME) were boosted once monthly intradermally with alternating 10 μg of sCD30L-Fc in Ribi adjuvant (Ribi Immunochem Research, Hamilton, MT) or 1×10^6 CV-1/EBNA cells expressing human CD30L for four injections. One week after the fourth boost, peroxidase dot-blot assays showed a serum titer of anti-CD30L antibody. One week later, animals were boosted with 5 μg of sCD30L-Fc plus 2×10^6 CV-1/EBNA cells expressing human CD30L into the tail vein. Three days later, spleens were removed and spleen cells were fused with the X63-Ag8.653 mouse myeloma cell line by standard methods using a 50% polyethylene glycol/dimethyl sulfoxide solution (Sigma). Hybridoma cultures were established in 96-well plates (Costar, Cambridge, MA). Ten days later, culture supernatants were screened in the primary screen procedure by an antigen capture assay using ^{125}I-labeled sCD30L-Fc. Briefly, 96-well plates were coated overnight with goat anti-mouse serum (Zymed, San Francisco, CA), blocked with 3% bovine serum albumin (Sigma), and incubated with 50 μl of culture supernatant for 1 hour at room temperature. After three washes with PBS, plates were incubated with ^{125}I-labeled sCD30L-Fc for 1 hour and were then washed with PBS before placing onto films for overnight exposure. Positive wells were examined for Fc reactivity with human IgG and human sCD27L-Fc by performing avidin-biotin complex (ABC) and dot-blot assays. One remaining positive culture well was rescreened for reactivity with human sCD30L-Fc and cloned. The positive supernatant was also examined by flow cytometry using CD30L-transfected CV-1/EBNA cells and phorbol myristate acetate/ionomycin-activated peripheral blood T cells. Large quantities of the human anti-CD30L M80 (mouse IgG_{2b} isotype) MAb were purified from spent bulk culture supernatants from hybridoma cells grown in roller bottles, as previously described.[12] Purified M80 MAb was stored frozen in 1 mg/ml aliquots at −20°C in 0.05 mol/L citrate buffer (pH 7.0).

MAbs Ber-H2/CD30, UCHL1/CD45RO, and KP1/CD68 were purchased from Dako Corp. (Santa Barbara, CA). Leu4/CD3, Leu3a/CD4, Leu12/CD19, and LeuM1/CD15 MAbs were obtained from Becton Dickinson (Mountain View, CA). The MAb M67 (anti-CD30) had been generated against CD30-Fc as described before.[12] The anti-CD40 MAb M2 and anti-CD40L MAb M90 (kindly provided by Dr. Richard Armitage, Immunex) and anti-CD40 MAb 89 (kindly provided by Dr. J. Banchereau, Centre de Recherche Schering-Plough, Dardilly, France) were used as previously described.[22–24] In all instances, isotype-matched control antibodies were used (Biosource International, Camarillo, CA).

Flow Cytometry

CD30L surface expression was examined by indirect immunofluorescence and flow cytometry as previously described.[11,13] Briefly, 1×10^6 cells/ml were preincubated with 100 μg/ml human IgG_1 (Sigma) in PBS containing 0.02% NaN_3 and 10% goat serum (Gibco) for 30 minutes at 4°C to prevent nonspecific binding of staining reagents. CD30-Fc, human IgG_1, and human IL-4R-Fc were biotinylated as described.[25] Cells were incubated with 10 μg/ml biotinylated CD30-Fc, IgG_1, or IL-4R-Fc as well as with 5 μg/ml of the indicated MAbs for 60 minutes at 4°C. After repeated washings in PBS containing 0.02% NaN_3, streptavidin-phycoerythrin (PE; Becton Dickinson, Mountain View, CA) or goat-anti mouse IgR-PE (Biosource International) were added for 60 minutes at 4°C for Fc staining or MAb staining, respectively. After two washes in PBS plus NaN_3 staining, intensity was estimated using FACScan (Becton Dickinson, Sunnyvale, CA). A minimum of 5000 cells were analyzed for each sample. Cells stained with unconjugated or biotinylated IgG_1, IL-4RFc, or isotype-matched control MAbs were included in this analysis to determine background fluorescence.

Immunohistochemistry and Immunocytochemistry

HD tissue samples included surgically removed lymph nodes (n = 6) and needle biopsy material

(n = 16) of consecutive, previously untreated HD patients and were obtained from the Division of Pathology, Centro di Riferimento Oncologico, and the Department of Medical Oncology and Applied Molecular Biology, Robert-Rössle Cancer Center. Initially, all primary lymph node tissues were characterized immunohistochemically using a panel of antibodies including B-cell- and T-cell-associated markers, as previously described.[26,27] Nonmalignant lymphoid specimens included reactive lymph nodes (n = 8) and tonsils (n = 5).

Some of the tissue samples were snap-frozen in liquid-nitrogen-cooled isopentane or were freshly dissected to single-cell suspensions. A three-step ABC technique or the alkaline-phosphatase anti-alkaline-phosphatase (APAAP) method was used for immunodetection as previously reported.[28,29] Immunostaining was either performed manually or on an automated staining instrument (Ventana 320, Ventana Medical Systems, Tucson, AZ) using 10 μg/ml primary antibody and the Ventana detection kit 250–001 with isotype-matched biotinylated secondary antibodies. The procedure was performed as recommended by the manufacturer's guidelines using DNAse2 digestive pretreatment to increase specific signal and diaminobenzidine as substrate. Frozen sections or single-cell suspensions from HD and reactive lymphoid tissues were stained manually using the APAAP method. For CD30L staining, cell smears or cytospins were formalin fixed for 10 minutes, dehydrated, and stored at −80°C until use. Before immunostaining, fixed cell preparations were treated with 0.33 mg/ml trypsin (Sigma) for 10 minutes. In control experiments, the primary antibody was replaced by an irrelevant, isotype-matched MAb or chromogen only. The staining intensity was semiquantitatively scaled as follows: −, no staining; +, weak staining, ++, moderate staining; and +++, strong staining.

Proliferation Assay

To ensure functional activity of CD30L expressed by granulocytes the $CD30^+$ HDLM-2 and KARPAS 299 cell lines (1×10^5 cells/ml) were cultured in the presence of titrations of fixed pure granulocytes on flat-bottomed 96-well microplates. Granulocytes were fixed with 0.5% paraformaldehyde as described.[30] Control experiments were performed in the presence of a 50-fold excess of CD30-Fc to block CD30/CD30L interactions, as previously described.[12,13] After 72 hours, cultures were pulsed with 1 μCi/well [^{3}H]thymidine ([^{3}H]TdR, 25 Ci/mmol; Amersham, Airlington Heights, IL) for 6 hours. Cells were harvested and incorporated cpm were determined by tritium-sensitive avalanche gas ionization using a Matrix 96 beta counter (Packard, Meriden, CT). Results were expressed as the mean cpm ± SD [^{3}H]TdR incorporation of triplicate cultures.

Results

Detection of Surface Expression of Recombinant and Natively Expressed CD30L

CV-1/EBNA cells were transfected either with vector alone (CV-1/HAV) or the human full-length CD30L cDNA (CV-1/CD30L). We have previously observed expression of CD30L by activated peripheral blood T cells and Burkitt-like lymphoma cells (eg, DG-75, U698M).[13] CV-1/HAV cells transfected with the control vector cDNA failed to stain with M80 MAb or biotinylated CD30-Fc (Figure 1, A and B). In contrast, as shown in Figure 1, C and D, CV-1/EBNA cells transfected with the human CD30L cDNA exhibited specific surface expression of CD30L by indirect flow cytometry using the M80 MAb or biotinylated CD30-Fc. Activated T cells strongly expressed CD30L, whereas resting T cells did not (Figure 1, E to H). Also for the EBV^- Burkitt lymphoma cell line DG-75 and B-NHL cell line U698M membrane expression of CD30L was confirmed when anti-CD30L MAb M80 was used (Figure 1, I and J). The CD30L-non-expressing HD-derived cell line L-428 failed to react with anti-CD30L MAb M80 (Figure 1, K and L). Binding of M80 MAb to the $CD30L^+$ cells was abolished in the presence of an excess of soluble CD30-Fc, confirming specificity of M80 for CD30L (data not shown). Similarly, cytospin preparations of CD30L-expressing CV-1/EBNA cells and activated peripheral blood T cells strongly bound anti-CD30L M80 MAb (Figure 2). In contrast, CV-1/EBNA cells transfected with vector alone and resting T cells failed to react with this reagent (data not shown). Thus, the M80 anti-CD30L MAb specifically reacts with membrane-bound CD30L as shown by flow cytometry and immunocytochemistry.

CD30L Expression by Nonmalignant, Reactive Lymphoid Tissues

Frozen sections of reactive tonsils and lymph nodes (n = 13) exhibited negligible amounts of $CD30L^+$ cells. In this regard, it should be noted that, for yet unknown reasons, frozen section immunohistochemistry appeared not to be a valid tool for evaluating

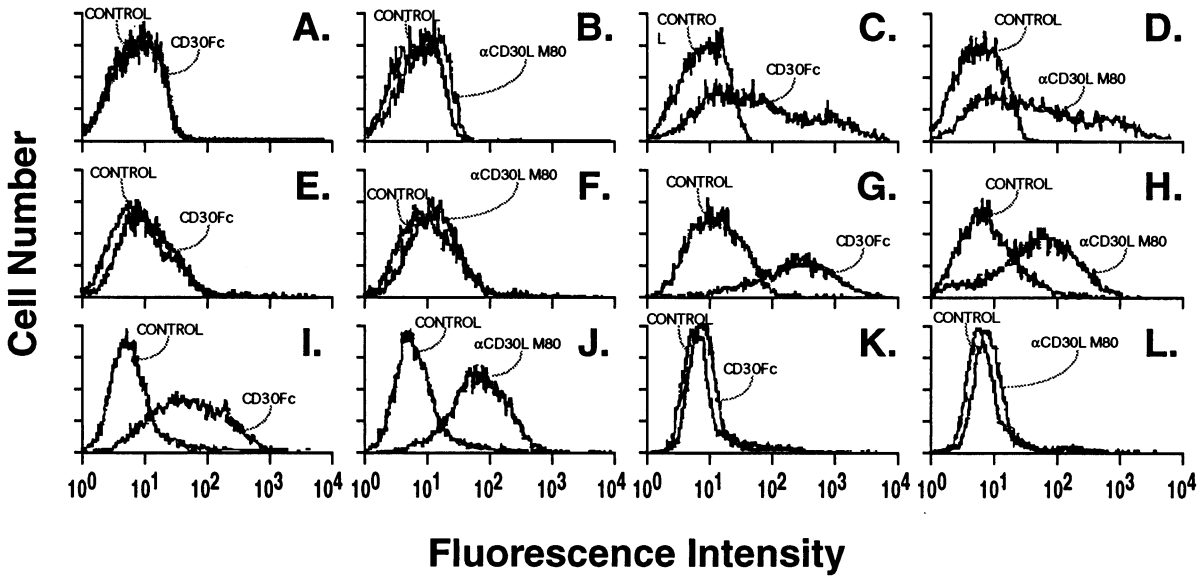


Figure 1. *The M80 anti-CD30L MAb reacts with recombinant and native CD30L. CV-1/EBNA cells expressing either the vector alone* (A *and* B: *CV-1/HAV) or the human full-length CD30L cDNA* (C *and* D: *CV-1/CD30L), resting T cells* (E *and* F*F), T cells* (G *and* H) *activated with phorbol myristate acetate and ionomycin for 24 hours, CD30L$^+$ DG-75* (I *and* J) *and CD30L$^-$ L-428* (K *and* L) *were stained with either 5 μg/ml biotinylated CD30-Fc* (A, C, E, G, I, *and* K) *or the anti-CD30L MAb M80* (B, D, F, H, J, *and* L) *developed by streptavidin-PE or PE-conjugated goat anti-mouse antibody (CD30-Fc and αCD30L M80), respectively. Background fluorescence was determined by staining with biotinylated IgG_1 or irrelevant control-Fc and isotype-matched (IgG_{2b}) control MAb and flow cytometry (Control). Analysis was performed on a FACScan with a minimum of 5000 cells examined for each sample. Results are presented as histograms, and the fluorescence intensity is shown in log_{10} intervals. Data are representative of three independent experiments performed.*

CD30L expression with the M80 reagent. Conversely, by using freshly prepared single-cell suspension from tonsils and lymph nodes, the anti-CD30L MAb M80 displayed specific reactivity with a number of cell types. CD30L was expressed by a fraction of lymphocytes (15 to 35%) and by the great majority (>90%) of histiocytes and granulocytes (Table 1). CD30L$^+$ lymphocytes were mainly identified as T cells based on CD3$^+$ and CD40L$^+$ co-expression (data not shown). A few B cells were also stained with the anti-CD30L MAb M80 (data not shown). Staining of histiocytes and granulocytes was stronger as compared with that of lymphocytes (Table 1). Lymphoid cells were stained in a localized granular cytoplasmic or dot-like paranuclear fashion, whereas histiocytes and granulocytes displayed a diffuse and usually inhomogeneous granular staining pattern in their cytoplasm (Figure 3 and data not shown).

Immunocytochemical Analysis of CD30L Expression in Single-Cell Suspension of HD-Involved Tissues

H-RS cells expressed CD30L in all cases of HD studied (n = 22), which covered all subtypes with nodular sclerosing (n = 12), mixed cellularity (n = 7), lymphocyte depletion (n = 1), and nodular lymphocyte predominance (LP; n = 2; Table 1 and Figure 3). CD30L staining in H-RS cells was usually weak, consistently granular, and homogeneously dispersed in the cytoplasm for all subtypes of HD except the LP subtype. In the LP subtype, the H-RS cells showed a homogeneous cytoplasmic staining (Figure 3, C, F, and I). Analysis of the frequency of H-RS cells expressing CD30L revealed 60 to 70% of M80 positively labeled H-RS cells regardless of the histological subtype of HD and of the H-RS phenotype involved (ie, CD30$^+$/CD40$^+$ and CD30$^-$/CD40$^+$). As shown in Figure 3, CD30L$^+$ H-RS cells from all histological subtypes strongly expressed the CD40 antigen (Figure 3, B, E, and H) whereas expression of CD30 (Figure 3, A, D, and G) was detected in H-RS cells of all HD subtypes except the nodular LP HD subform. The number of CD30L$^+$ bystander cells was much higher in HD-involved lymph nodes as compared with CD30L expression seen in reactive lymphoid tissues (approximately 80% *versus* 35%, respectively). The staining pattern of CD30L$^+$ bystander cells that surrounded H-RS cells was similar to that seen with cell suspensions obtained from nonmalignant lymphoid tissues, but the intensity of staining was usually different (Table 1). In addition, a number of small lymphoid cells interspersed among or surrounding H-RS cells and a few large lymphoblastoid cells were found to display

Table 1. *Summary of CD30L Expression by Hodgkin's Disease-Involved Tissues and Nonmalignant Reactive Lymphoid Tissue Examined in Single-Cell Suspensions*

Types of cell suspensions (number of cases)	CD30L immunoreactivity			
	H-RS cells	Lymphocytes	Histiocytes	Granulocytes
HD (n = 22)	+	+/++	++/+++	++/+++
Reactive tonsils (n = 5)		+	+/++	+/++
Reactive lymph nodes (n = 8)		+	+/++	+/++

Reactivity and intensity of staining was semiquantitatively scaled as follows: −, no staining; +, week staining; ++, moderate staining; and +++, strong positive staining. HD cases covered all of the main subtypes and included 12 cases of nodular sclerosis, 7 cases of mixed cellularity, 1 case of lymphocyte depletion, and 2 cases of nodular lymphocyte predominance.

a clear surface expression of the CD30 antigen (Figure 4). $CD30^+$ T lymphocytes were present in almost all HD cases examined regardless of the histological subtype, even though quantitative variations among histological subtypes (ie, up to 50% in non-LP HD cases and usually less than 15% in LP HD cases) were found (data not shown). These T cells also co-expressed CD3, CD4, and CD45RO (Figure 4 and data not shown).

Functional Expression of CD30L by Granulocytes

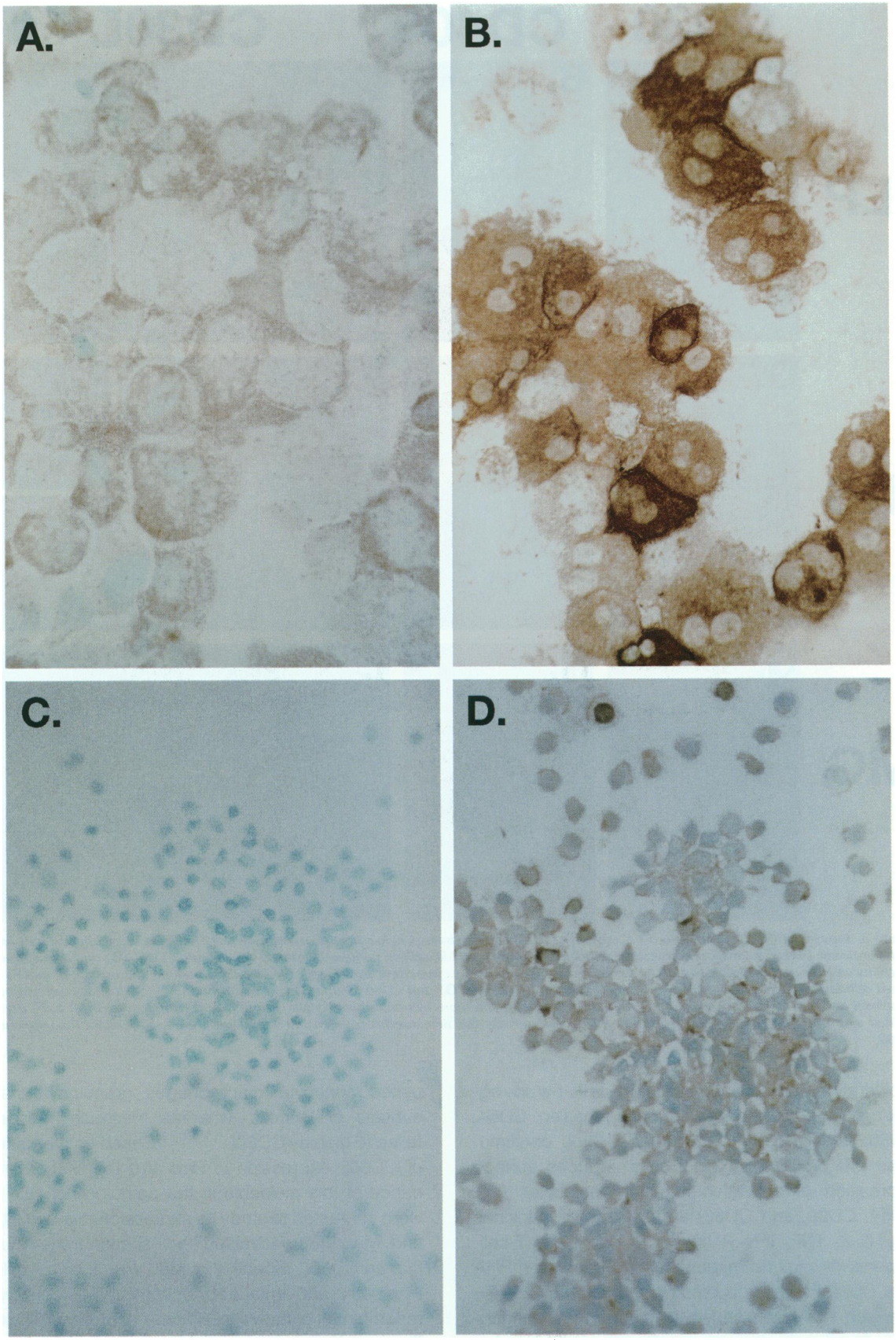
Granulocytes were detected as a strong source of CD30L in HD-involved tissues (Figure 3). Also, reactive pleural effusions of three HD patients were found to contain large numbers of granulocytes strongly stained with the CD30L M80 MAb (Figure 5A). Similarly, peripheral blood granulocytes obtained from healthy donors (Figure 5B) and from HD patients (not shown) constitutively expressed CD30L. To examine whether the CD30L on the surface of these cells was functional on $CD30^+$ target cells, titrations of 0.5% paraformaldehyde-fixed granulocytes were added to 1×10^5 $CD30^+$ responder cells/ml, known to proliferatively respond to membrane-bound recombinant CD30L.[11,12] CD30L-mediated biological responses were detected using the [^{3}H]TdR incorporation assay. As shown in Figure 6, $CD30L^+$ granulocytes induced a dose-dependent proliferation of the HD-derived cell line HDLM-2 and inhibited growth of the $CD30^+$ anaplastic large cell lymphoma cell line KARPAS 299, as expected from previous experiments made with recombinant CD30L.[11,12] Specificity was confirmed by addition of a 50-fold excess of CD30-Fc fusion protein to block CD30L-mediated responses (Figure 6). Thus, $CD30L^+$ granulocyte express a functional surface molecule, capable of interacting with $CD30^+$ cells.

Discussion

The development of antibodies against H-RS cells by using HD-derived cell lines as immunogen has facilitated the identification of the CD30 antigen.[1,3,6] Subsequently, expression of the CD30 antigen was found to be associated with HD by demonstrating that H-RS cells of more than 90% of mixed cellularity, nodular sclerosing, and lymphocyte depletion cases and approximately one-third of LP cases expressed this surface antigen (reviewed in Refs. 31 and 32). For these reasons, the CD30 antigen has been viewed as a suitable tool for diagnosis, immunoimaging, and immunotherapy of HD patients.[33] The CD30 antigen is, however, neither expressed in a cell lineage-restricted fashion nor is it specific for H-RS cells.[34] For instance, in the immune system, CD30 plays a role as a lymphocyte activation antigen, mainly for a memory T cell subset exhibiting the $CD4^+/CD45RO^+$ phenotype.[35]

Since its molecular cloning, CD30 has been identified as a member of the TNF/nerve growth factor receptor superfamily by sequence homology.[7] In parallel, the counterstructure of CD30, termed CD30L, was molecularly characterized as a type II membrane protein with pleiotropic cytokine-like activities.[11] Both studies have indicated that CD30 may act as a cytokine receptor on H-RS cells involved in their survival, proliferation, or differentiation.[34] CD30L is known to stimulate cytokine secretion by H-RS cells (eg, IL-6, TNF, LT-α) and to enhance

Figure 2. *The M80 anti-CD30L MAb stains cells expressing recombinant membrane-bound CD30L by immunocytochemistry. CV-1/EBNA cells were transfected with the human full-length CD30L cDNA* (**A** *and* **B**). *After 48 hours, cytospins were prepared and formalin fixed for 10 minutes. In addition, formalin-fixed cytospin preparations of phorbol myristate acetate/ionomycin-activated T cells for 24 hours* (**C** *and* **D**) *were used. Immunostaining was performed on an automated staining instrument using either 10 μg/ml control MAb* (**A** *and* **C**) *or 10 μg/ml M80 anti-CD30L MAb* (**B** *and* **D**), *biotinylated secondary antibody, and diaminobenzidine as substrate. Original magnification, ×250 or ×400.*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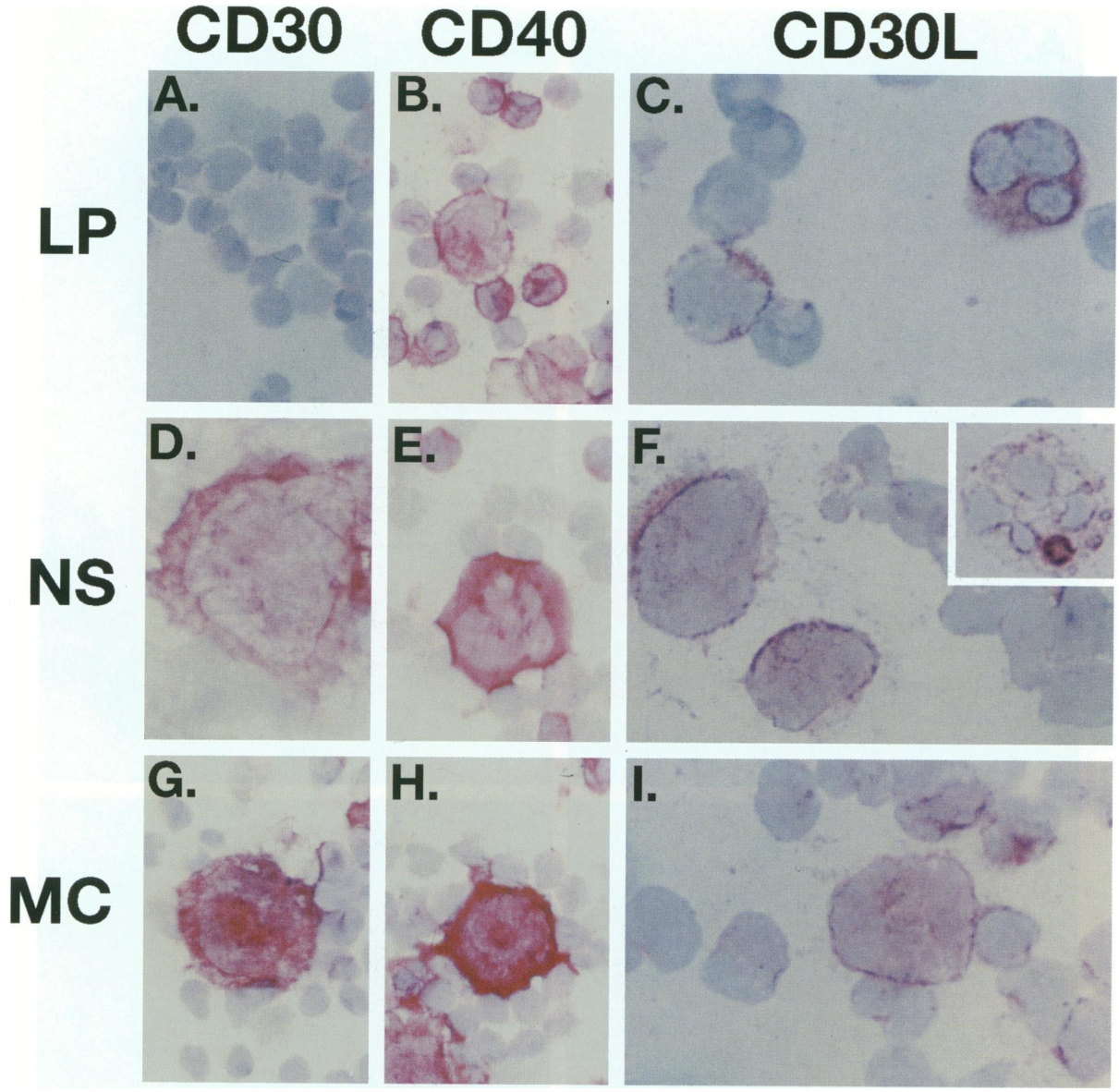

Figure 3. *Staining of CD30L in single-cell suspensions of HD-involved tissues. Single-cell suspensions were prepared from surgically removed lymph nodes or needle biopsy materials of consecutive untreated HD patients. H-RS cells in nodular LP are CD30⁻* (A) *and CD40⁺* (B) *and display specific reactivity with the anti-CD30L MAb M80 by demonstrating homogeneous cytoplasmic staining* (C). *H-RS cells in nodular sclerosis* (D, E, *and* F) *and mixed cellularity* (G, H, *and* I) *subtypes strongly express CD30* (D *and* G) *and CD40* (E *and* H). *By immunostaining, CD30L was detected in the cytoplasm and was consistently weak and granular* (F *and* I). *In addition, bystander lymphocytes* (F *and* I) *and granulocytes* (inset *in* F) *are also stained with anti-CD30L MAb M80. The APAAP immunostaining and hematoxylin counterstain techniques were used. Original magnification,* ×*400.*

proliferation and activation of these cells by up-regulating cell-contact-dependent antigens (eg, CD54, CD80, and CD86).[14,16] Similarly, CD30L co-stimulates the proliferation of T cells, induces surface expression of activation/adhesion molecules (eg, CD54, CD80, and CD86) and release of cytokines (eg, IL-2, TNF, IFN-γ) known to boost the T-cell-dependent immune response.[11,15] Based on mRNA and CD30-Fc surface binding studies, CD30L expression has been shown in mitogen- or antigen-activated T cells, lipopolysaccharide, or IFN-γ-stimulated monocytes/macrophages, granulocytes, and subsets of B cells, whereas mesenchymal cells failed to display CD30L.[11,13–15] Based on the pattern of CD30L expression *in vitro* and the likely significance of this molecule in the pathology of HD, we were interested to study its expression *in vivo*. To this end, MAb M80 was raised against membrane-bound human CD30L. Specifity of M80 was shown by flow cytometry and immunocytochemistry of cells with recombinant or native membrane-expressed CD30L. The specificity of the M80 MAb against CD30L was

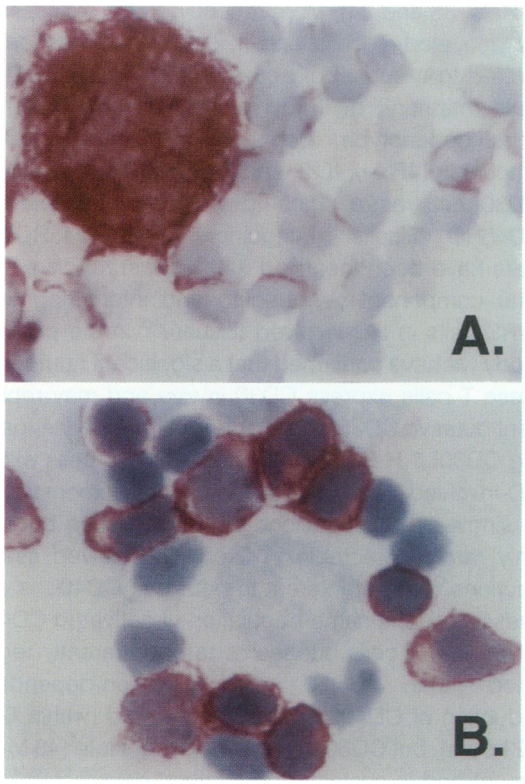

Figure 4. *CD30 reactivity in single-cell suspension of mixed cellularity HD-involved tissue. Many bystander lymphocytes surrounding a CD30$^+$ H-RS cell react with anti-CD30 MAb Ber-H2* (**A**). *In addition, several CD45RO-stained lymphocytes surround an unreactive H-RS cell* (**B**). *The APAAP immunostaining and hematoxylin counterstain techniques were used. Original magnification,* ×*630.*

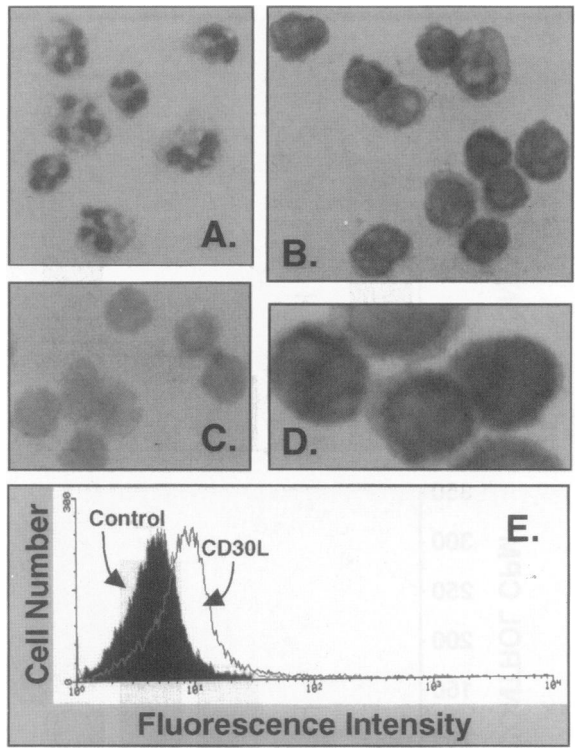

Figure 5. *Strong surface expression of CD30L by granulocytes. Granulocytes obtained from a reactive pleural effusion of a HD patient were stained with Pappenheim* (**A**) *or an irrelevant isotype-matched control MAb* (**B**) *and the CD30L MAb M80* (**C** *and* **D**) *using the APAAP immunostaining technique. Original magnification,* ×*400* (**A** *to* **C**) *or* ×*630* (**D**). *Blood granulocytes obtained from a healthy blood donor* (**E**) *were stained with 5 μg/ml anti-CD30L MAb M80 and followed by PE-conjugated goat anti-mouse antibody and analyzed by flow cytometry* (**line**). *Background fluorescence was determined by staining with isotype-matched* (IgG_{2b}) *control MAb* (**solid**). *Analysis was performed on a FACScan with a minimum of 5000 cells analyzed for each sample. Results are presented as histograms with fluorescence intensity in* log_{10} *intervals.*

confirmed by an excess of CD30-Fc fusion protein as specific blocking reagent for CD30/CD30L interactions. M80 MAb was then used to investigate expression of CD30L in nonmalignant, reactive lymphoid tissues and tissues involved in HD. M80 MAb recognized native and recombinant membrane-bound CD30L by immunostaining of cytospins and by flow cytometry analysis of single-cell suspensions, but for yet unknown reasons failed to detect CD30L in frozen sections of nonmalignant and malignant lymphoid tissues. Using single-cell suspensions of these tissues, our findings demonstrate that CD30L is expressed by lymphocytes, granulocytes, and histiocytes with a granular cytoplasmic and surface (flow cytometry) staining pattern. The surface density of CD30L was highest on granulocytes and histiocytes where its physiological role remains still unclear. A subset of CD4$^+$/CD45RO$^+$ T cells are the major normal cell source expressing CD30, but nonhematological cells, including endothelial cells, lack CD30 expression.[2,3,5,6,35] On the other hand, we were able to show that native CD30L expressed by resting and activated granulocytes is functionally active in transmitting specific signals on CD30$^+$ cell lines. Therefore, the interaction of CD30L$^+$ granulocytes with various sources of endothelial cells is currently being investigated by us to demonstrate a possible role of CD30/CD30L interactions in transendothelial migration of granulocytes.

Even though previous *in vitro* data obtained with cultured H-RS cells did not support the idea that the CD30/CD30L interaction was operating in autocrine growth stimulation,[12,13] we were now able to show that CD30L is immunodetectable in the majority of H-RS cells of all HD cases investigated. The expression of CD30L in H-RS cells was characteristic in that a weak granular staining pattern was consistently found in the cytoplasm. In HD, tumor cell growth may be mediated by either unbalanced or deregulated secretion of cytokines and growth factors produced in an autocrine or paracrine fashion by either H-RS cells themselves or surrounding bystander cells.[18] These observations, along with previous data on

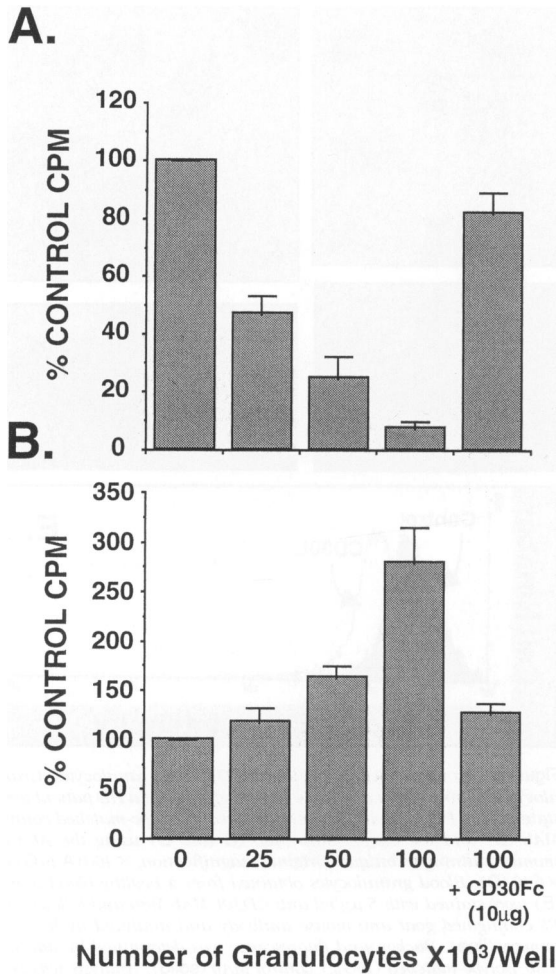

Figure 6. *Functional expression of CD30L on granulocytes. Biological activity of the CD30L⁺ granulocytes was assessed on CD30⁺ responder cell lines. A titration of paraformaldehyde-fixed granulocytes was incubated with 1 × 10⁵ KARPAS 299* (**A**) *or HDLM-2* (**B**) *cells for 72 hours. Cultures were pulsed with 1 μCi/ml β³H]TdR for the last 6 hours of culture. Tritiated thymidine incorporation was determined by β-scintillation counting and expressed as cpm ± SD. Control experiments were performed in the presence of a 50-fold excess (10 μg/well) of the blocking CD30-Fc. Results are representative of three independent experiments.*

CD30L expression by hyper-reactive normal cells accumulating in HD-involved areas, have pointed to the engagement of the CD30L in a paracrine growth-stimulatory loop in HD.[14] It seems likely that the CD30⁺ H-RS cells interact with surrounding activated CD30L⁺ bystander cells such as T cells and granulocytes.[34] In addition, identification of CD30L on the majority of primary H-RS cells further points to the functional role of this molecule in HD.

Previous studies on CD30 distribution in non-neoplastic tissues have indicated that the CD30 surface expression is mainly restricted to mitogen- or antigen-driven and virally transformed lymphocytes, natural killer cell clones, and a small subset of large lymphoid cells in lymph nodes and tonsils (reviewed in Refs. 33 and 34). More recently, CD30 expression by activated T cells and T cell clones has been related to a subset of activated CD4⁺/CD45RO⁺ T cells (memory) or T cell clones with mainly Th-2 immunophenotype, capable of secreting large amounts of IFN-γ, IL-2, and IL-5.[15,35–39] In addition, CD30 acts as a signal-transducing molecule for CD45RO⁺ T cells.[35] Intriguingly, CD4⁺/CD45RO⁺ T cells have been identified as the main reactive cellular component surrounding and interacting with H-RS cells in HD-involved tissues.[40] In the present study we have confirmed that a significant number of these T cells express CD30 *in vivo* and may represent putative CD30⁺ cellular targets possibly engaging CD30L⁺ H-RS cells. These CD30⁺ T cells within HD-involved lymphoid tissues can be demonstrated in single-cell suspensions of tumor tissues but are only rarely detectable in fixed HD-involved tissue sections.[2] It is of interest to note that CD40L is expressed on the same population of activated CD4⁺/CD45RO⁺ T cells but seems to be differently regulated than CD30.[22–24] Activation-dependent induction of CD40L expression is rapid (within 6 to 12 hours), but CD30 expression occurs late (48 to 96 hours) and is sustained for several days.[5,15,35,41,42] These data suggest that CD40L and CD30 play distinct but most likely coordinated roles in the processes of activation and cell-contact-dependent interactions of these CD4⁺/CD45RO⁺ T cells. We have previously shown that high numbers of CD40L-expressing CD4⁺ T cells accumulate in HD tissues and might functionally engage CD40 expressed by H-RS cells.[22–24] Therefore the CD30/CD30L and CD40/CD40L interaction could be viewed as critical element for H-RS cell/T cell interactions using several pathways.[34] It might well be that activated CD4⁺ T cells bind H-RS cells via the CD40L/CD40 interaction and subsequently tighten their cellular interaction by a CD30/CD30L-mediated cell-cell adhesion. These cellular interactions are further strengthened by other adhesion/activation molecules expressed by H-RS cells (eg, ICAM-1/CD54, LFA-3/CD58, B7–1/CD80, and B7–2/CD86) and their specific counter-receptors on activated T cells (eg, LFA-1/CD11a, LFA-2/CD2, and CD28).[22,31,43–49] This hypothesis is further supported by previous findings demonstrating that recombinant and native CD30L and CD40L are able to enhance surface expression of adhesion/activation molecules when their respective counter-structures (CD30 and CD40) had been engaged on either H-RS cells and/or T cells.[15,16,22]

The presence of CD30, CD40, and CD30L on H-RS cells and sequential induction of CD40L, CD30L, and CD30 on activated CD4⁺/CD45RO⁺ T

cells may allow for amplification of a cross-stimulatory cascade of cell-contact-dependent interactions among these two cell types contributing to the typical cellular reaction seen in HD.[17] Various cytokines (eg, IL-1, IL-2, IL-5, IL-6, IL-7, IL-8, IL-9, IL-12, TNF, LT-α, CD27L/CD70, CD40L, macrophage colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, transforming growth factor-β, and leukemia inhibitory factor) and membrane-anchored activation/adhesion molecules (eg, CD25, CD30, CD40, CD54, CD71, CD80, and CD86) have been found to relate to distinct clinical or pathophysiological features of HD.[18] In addition, recombinant CD30L has mitogenic activity for some of the cultured H-RS cell lines and induces cellular activation that results in enhanced cytokine production (eg, IL-6, TNF, and LT-α).[12,16] The CD30/CD30L interaction thus seems to be part of the deregulated cytokine network involved in the cellular activation cascade. Whether the expression of CD30L on primary H-RS cells might also contribute to the cellular growth in HD by triggering an autocrine stimulatory loop is presently under investigation.

Taken together, the detection of CD30L expression by H-RS cells and surrounding bystander cells, ie, lymphocytes, granulocytes, and histiocytes, suggests functional involvement of CD30/CD30L interactions in growth, survival, and cellular activation of H-RS cells. The direct cell-cell contact of H-RS cells with surrounding bystander cells might be mediated through CD30/CD30L binding. Thus, the CD30L actions seem to be a critical component in the pathobiology of HD.

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