

## **CDw60: A Marker for Human CD8<sup>+</sup> T Helper Cells**

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### **Summary**

The existence of helper cells among the CD8<sup>+</sup> T cell subset has been recognized for a long time. However, the phenotype of these cells has remained elusive. In this study, we provide evidence that the expression of the CDw60 antigen on human CD8<sup>+</sup> T cell allows one to distinguish between CD8<sup>+</sup> T helper cells and CD8<sup>+</sup> T cells with cytotoxic and suppressor capacity. CDw60 monoclonal antibodies (mAb) recognize the 9-*O*-acetylated disialosyl group on ganglioside GD<sub>3</sub> expressed on 20–40% of CD8<sup>+</sup> cells. By use of the direct and indirect mAb-rosetting technique, we were able to isolate the CDw60<sup>+</sup>CD8<sup>+</sup> and CDw60<sup>-</sup>CD8<sup>+</sup> cells at high purity. The alloantigen-specific cytotoxic activity of CD8<sup>+</sup> cells resided entirely in the CDw60<sup>-</sup> population. Helper and suppressor capacity of both CD8 subsets was assayed by the pokeweed mitogen-induced differentiation of B cells into immunoglobulin-secreting cells. These studies clearly indicate that the CDw60<sup>+</sup>CD8<sup>+</sup> subset provided substantial help to B lymphocytes, whereas the CD8<sup>+</sup> cells with the CDw60<sup>-</sup> phenotype were suppressing B cell differentiation. Both subsets produced similar amounts of interleukin 2 (IL-2) after stimulation with phytohemagglutinin. Activation with phorbol myristate acetate in combination with Ca-ionophore induced IL-4 secretion in both populations, but preferentially in the CDw60<sup>+</sup> subset, whereas the vast majority of interferon  $\gamma$  was produced by the CDw60<sup>-</sup>CD8<sup>+</sup> cells. When used in combination with other markers, CDw60 may prove to be useful in defining CD8<sup>+</sup> subsets with reciprocal functional activities.

**H**uman CD8<sup>+</sup> T lymphocytes are a functionally heterogeneous population that is characterized by the recognition of antigenic peptides bound to MHC class I molecules. They comprise antigen-specific cytotoxic progenitor and effector cells and lymphocytes capable of suppressing both T and B cell responses (1). In addition, helper activity has been reported for CD8<sup>+</sup> T cells both in humans (2) and in mice (3–5). Several attempts have been made to correlate the different functional capacities of CD8<sup>+</sup> T cells with the expression of a distinct cell surface structure. The mAb 9.3 recognizing the CD28 antigen (6) and the mAb Leu-15 reacting with the CD11b antigen (7) have been widely used as a means to distinguish between cytotoxic and suppressor lymphocytes within the CD8<sup>+</sup> T cell subset. However, surface markers delineating CD8<sup>+</sup> Th cells have not been identified thus far, and the proportion of these cells in the CD8<sup>+</sup> T cell subset is still unknown. The phenotypic and functional characterization of CD8<sup>+</sup> Th cells may add important new aspects to the analysis of lymphocyte subsets for diagnostic purposes. Here we provide evidence that the CDw60 antigen is a suitable marker for human CD8<sup>+</sup> Th cells. The CDw60 antigen has recently been identified as the *O*-acetylated disialosyl group which is expressed on certain gangliosides (8) and possibly in association with leukocyte membrane proteins (9, 10). It was found at different concentrations in lipid extracts of leukocytes of all hematopoietic differentiation lineages (11).

Surface expression of CDw60, however, appears to be restricted to subsets of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and to platelets (12). The CDw60<sup>+</sup> subset of CD4<sup>+</sup> T cells has already been shown to contain most of the helper activity for the mitogen-driven B cell differentiation (9). A particularly high frequency of CDw60<sup>+</sup> cells was detected in normal and rheumatoid synovial T cells (13), in cutaneous psoriatic T cells (10) and in leukemic cells from patients with cutaneous T cell lymphoma (14). The biological role of membrane structures containing the CDw60 determinant remains elusive. They might serve as ligands for yet unidentified lectinlike adhesion molecules. Furthermore, the CDw60 structure is considered the surface component of a separate activation pathway in T lymphocytes since CDw60 mAbs can provide costimulatory signals in the activation of both mature T lymphocytes (9, 15) and thymus cells (16).

### **Materials and Methods**

**Antibodies.** The following murine mAbs were prepared in our laboratory and were used to isolate and characterize T cell subpopulations: M-T310 (CD4), M-T811 (CD8), M-T910 (CD2), M-M42 (CD14), and M-M522 (CD11b). mAb M-T32 was raised against the leukemic cells from a patient with chronic lymphocytic T cell leukemia. It was clustered together with three additional mAbs as CDw60 antibody at the IVth International Workshop and Conference on Human Leukocyte Differentiation Antigens (12).

The CD16 hybridoma B73.1 was kindly provided by B. Perussia (Jefferson Cancer Institute, Philadelphia, PA). mAbs were purified by block electrophoresis of concentrated hybridoma supernatants followed by gel chromatography on Sephacryl S 200 (Pharmacia, Freiburg, FRG). Purified mAbs were FITC-labeled by dialysis against 250  $\mu$ M FITC (Fluka, Neu-Ulm, FRG) in carbonate buffer pH 9.0. PE-labeled goat F(ab')<sub>2</sub> anti-mouse IgM and FITC-labeled CD20 mAb were purchased from Dianova (Hamburg, FRG).

**Isolation of Cell Subpopulations.** PBMC were prepared by Ficoll-Hypaque (Pharmacia) density centrifugation of heparinized blood samples obtained from healthy volunteer donors. T lymphocytes were isolated by rosetting with sheep erythrocytes treated with aminoethyl isothiuronium bromide (AET, Serva, Heidelberg, FRG) and Ficoll density centrifugation as described (17) (E<sup>+</sup> cells). The interphase cells were completely depleted of T cells by a second round of rosetting with AET-treated sheep erythrocytes followed by centrifugation through Percoll (Pharmacia) of 1.077 g/ml density. The interphase cells (non T cells) were used as a source of B cells and accessory cells. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were prepared by removal of the CD8<sup>+</sup> and CD4<sup>+</sup> cells, respectively, from the T cell population by a direct mAb rosetting technique (DART) using bovine erythrocytes (BRBC) coated with CD8 or CD4 mAbs by the CrCl<sub>3</sub> method essentially as described (17). Monocytes were isolated from PBMC as rosettes with BRBC coated with the CD14 mAb M-M42. CDw60<sup>+</sup> and CDw60<sup>-</sup> cells were isolated from CD8<sup>+</sup> T cells by an indirect mAb rosetting technique (IART). CD8<sup>+</sup> T cells were first incubated with mAb M-T32, washed, and then rosetted with BRBC coated by the CrCl<sub>3</sub> method with a rabbit IgG specific for mouse IgM (Dako, Hamburg, FRG). The rosetted CDw60<sup>+</sup> cells were separated from the CDw60<sup>-</sup> cells by centrifugation through Percoll of 1.082 g/ml density. Purity of T cell subset preparations was in excess of 98% as revealed by two-color fluorescence analysis in flow cytometry.

**Analysis of Lymphocyte Populations with Two-color Fluorescence Flow Cytometry.** For two-color analysis, cells were first stained with 3  $\mu$ g/ml of the CDw60 mAb M-T32 followed by a PE-labeled goat F(ab')<sub>2</sub> anti-mouse IgM (Dianova). Subsequently, cells were incubated with FITC-labeled mAbs of IgG isotype. Flow cytometric analysis was performed on a FACScan<sup>®</sup> (Becton Dickinson & Co., Heidelberg, FRG).

Functional assays were performed as described below.

**Cell Cultures.** Unfractionated and separated populations of lymphocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, and antibiotics (all from Gibco, Karlsruhe, FRG) in round-bottomed microtiter plates (Costar, Heidelberg, FRG) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**T Cell Cytotoxicity.** Cytotoxic T cells were generated by incubation of 2 × 10<sup>7</sup> responder PBL with 2 × 10<sup>7</sup> irradiated (2,000 rad) allogeneic stimulator PBL in tissue culture flasks (50 ml, Costar) for 5 d. CD8<sup>+</sup> cells were prepared by depletion of CD4<sup>+</sup> cells using DART and then separated into CDw60<sup>+</sup> and CDw60<sup>-</sup> cells by IART as described above. After overnight incubation with 20 IU/ml of rIL-2 (kind gift of Hoffmann La Roche, Basel, Switzerland), the separated subsets were assayed at different E/T cell ratios in a 4-h cytotoxicity test. <sup>51</sup>Cr-labeled allogeneic stimulator cells cultivated previously for 3 d with 1  $\mu$ g/ml of PHA served as target cells as described (18). <sup>51</sup>Cr release was determined in the supernatants, and specific cytotoxicity was calculated according to the formula: Percent specific lysis = 100 × [(cpm test release - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release)].

**Helper Cell Assay.** The non T cell fraction was depleted of the

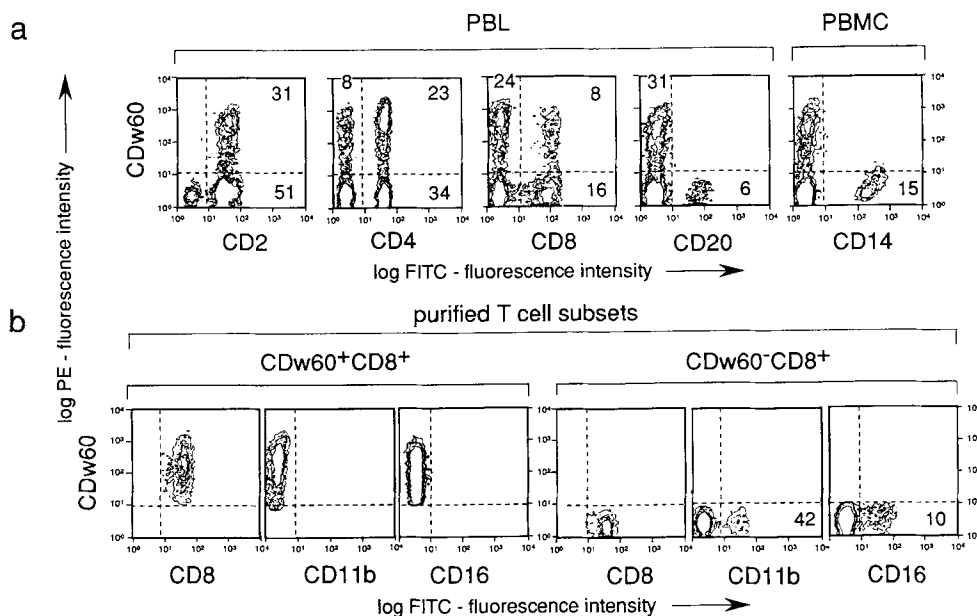
majority of monocytes by adherence to plastic for 1 h. The nonadherent cells contained about 5% monocytes, 30–50% B cells, <1% T cells and about 15% NK cells as verified by immunofluorescence staining with the lineage-specific mAbs CD14, CD20, CD2 and CD16. 5 × 10<sup>4</sup> cells of this population were incubated with 5 × 10<sup>4</sup> cells derived from the various isolated T cell subsets in a final volume of 200  $\mu$ l in round-bottomed microtiter culture plates (Costar). Differentiation of B lymphocytes and production of IgG was induced by cultivating with 50  $\mu$ g/ml of PWM (Gibco). Every cell combination was set up in triplicate. Culture supernatants were harvested after 7 d for determination of IgG secretion by an IgG-specific ELISA.

**Lymphokine Secretion.** 10<sup>5</sup> separated T cells were mixed with 10<sup>4</sup> isolated blood monocytes and cultured in a final volume of 200  $\mu$ l in round-bottomed microtiter plates. The cells were stimulated either with 1  $\mu$ g/ml PHA or with 100 ng/ml PMA in combination with 1  $\mu$ g/ml of 4-bromo calcium ionophore (Sigma, Deisenhofen, FRG) for 1, 2, and 4 d. Secreted IFN- $\gamma$  was determined with a RIA (Centocor, Malvern, PA); IL-2 and IL-4 were evaluated from the culture supernatants using ELISA detection systems (Genzyme, Munich, FRG) following the manufacturers advice.

## Results

**Immunofluorescent Staining of CDw60<sup>+</sup> PBL and Purification of CD8<sup>+</sup> Subsets.** The various subpopulations of PBL recognized by the CDw60 mAb M-T32 were analyzed by two-color fluorescence flow cytometry using FITC-labeled lineage-specific mAbs. Fig. 1 a gives the results of a representative experiment. All blood lymphocytes labeled by the CDw60 mAb were located within the CD2<sup>+</sup> T cell subset, whereas B cells and monocytes were negative. About 40% of the CD4<sup>+</sup> T cells and one third of the CD8<sup>+</sup> T cells expressed the CDw60 phenotype. To isolate the CD8<sup>+</sup> subsets according to their CDw60 expression, E<sup>+</sup> cells were first depleted of CD4<sup>+</sup> cells by rosetting with CD4 mAb-coated bovine erythrocytes. The remaining cells were >98% CD8<sup>+</sup> (data not shown). This population was separated into CDw60<sup>+</sup> and CDw60<sup>-</sup> cells by IART. The purity of the isolated subsets was >98% as demonstrated in Fig. 1 b. CD11b has been described as a selective marker for CD8<sup>+</sup> T suppressor cells (7). To see whether these cells are preferentially found in one of the CDw60-defined CD8<sup>+</sup> subsets, the isolated CDw60<sup>+</sup>CD8<sup>+</sup> and CDw60<sup>-</sup>CD8<sup>+</sup> populations were double stained with the CDw60 mAb M-T32 followed by PE-anti-mouse IgM Ab and with the FITC-labeled CD11b mAb. As can be seen from Fig. 1 b, CD11b-expressing CD8<sup>+</sup> T cells were exclusively found in the CDw60<sup>-</sup> subset. When the CDw60-separated CD8<sup>+</sup> T cell subsets were stained with a CD16 mAb, virtually all labeled cells appeared in the CDw60<sup>-</sup> subset (Fig. 1 b).

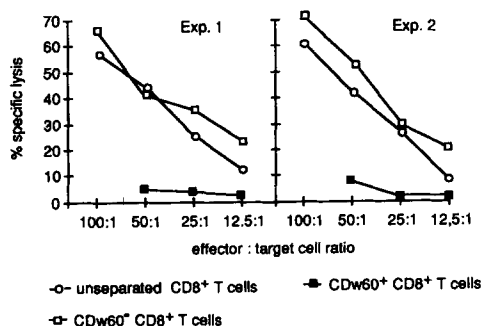
**Cytotoxic Capacity of CD8<sup>+</sup> T Cell Subsets.** Specific cytotoxicity of T cells is almost completely confined to the CD8<sup>+</sup> subset (19). To see whether expression of the CDw60 phenotype can be used as an additional marker for a more precise phenotypic identification of cytotoxic T cells, alloantigen-specific cytotoxic T cells were generated and subsequently separated into CDw60<sup>+</sup>CD8<sup>+</sup> and CDw60<sup>-</sup>CD8<sup>+</sup> subsets. When assayed in a 4-h cytotoxicity test against <sup>51</sup>Cr-



**Figure 1.** (a) Analysis of CDw60 expression on PBL by two-color flow cytometry. Cells were stained with the CDw60 mAb M-T32 followed by PE-labeled anti-mouse IgM and directly FITC-labeled subset-specific mAbs. Scale is  $\log_{10}$  fluorescence intensity. Numbers give the percentage of labeled cells in single gates. (b) Purity of isolated CDw60<sup>+</sup> and CDw60<sup>-</sup> subsets of CD8<sup>+</sup> T lymphocytes and expression of CD11b. The subsets were isolated by IART from purified CD8<sup>+</sup> blood T lymphocytes and stained with CDw60 mAb M-T32 followed by PE-labeled anti-mouse IgM and directly FITC-labeled CD8 or CD11b mAbs. Scale is  $\log_{10}$  fluorescence intensity. Numbers give the percentage of labeled cells in single gates.

labeled stimulator PHA blasts, the alloantigen-specific cytotoxicity was completely restricted to the CDw60<sup>-</sup> subset as shown in Fig. 2, which gives the results of two representative experiments. These data suggest the CDw60<sup>-</sup> phenotype as an additional marker for antigen-specific cytotoxic T cells.

**Helper Capacity of CD8<sup>+</sup> T Cell Subsets.** The helper activity of T cell subsets was tested by the PWM-induced and T cell-supported B cell differentiation into IgG-secreting cells. A cell population enriched for B cells (30–50%), containing about 5% monocytes and <1% T cells was mixed with different T cell subsets and cultivated in the presence of PWM. After 7 d, the amount of IgG secreted into the supernatant was determined by ELISA. Fig. 3 shows the results of four representative experiments. When T cells were separated into CD4<sup>+</sup> and CD8<sup>+</sup> cells, helper activity for IgG produc-

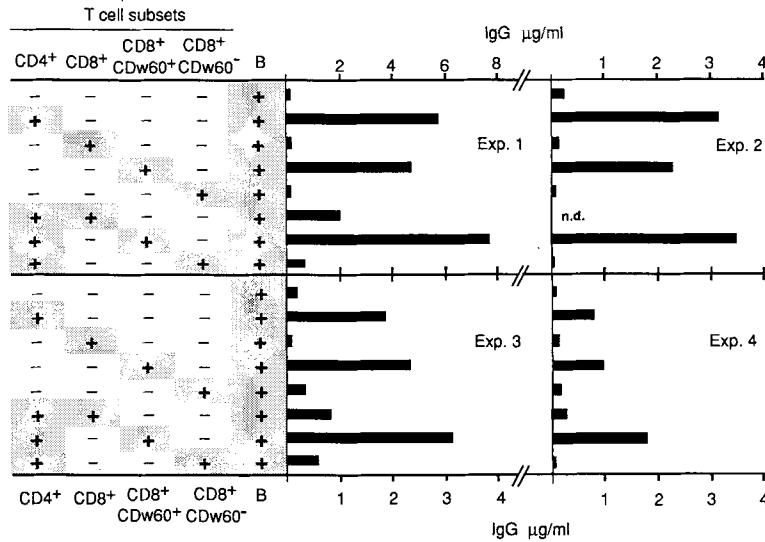


**Figure 2.** Cytotoxic capacity of CDw60-separated CD8<sup>+</sup> T cells. Cytotoxic T cells were generated by in vitro stimulation of PBL from two individual donors with allogeneic, irradiated PBL for 5 d. CD8<sup>+</sup> T cells were isolated, separated into CDw60<sup>+</sup> and CDw60<sup>-</sup> cells, and after overnight incubation with IL-2, assayed against <sup>51</sup>Cr-labeled stimulator 3-d PHA blasts in a 4-h <sup>51</sup>Cr-release test.

tion was preferentially found in the CD4<sup>+</sup> subset. However, when the CD8<sup>+</sup> T cell population was further split into CDw60<sup>+</sup> and CDw60<sup>-</sup> cells, it also became evident that the CD8<sup>+</sup>CDw60<sup>+</sup> subpopulation was capable of supporting the PWM-induced B cell differentiation, whereas the CD8<sup>+</sup>CDw60<sup>-</sup> cells were unable to help. So far, CD8<sup>+</sup> cells have been considered suppressors of the T cell-dependent mitogen-induced B cell differentiation in vitro (1). This finding was confirmed in our experiments.  $5 \times 10^4$  CD8<sup>+</sup> cells added to a mixture of  $5 \times 10^4$  enriched B cells and  $5 \times 10^4$  CD4<sup>+</sup> T cells markedly reduced the IgG production. Since CD8<sup>+</sup>CDw60<sup>+</sup> T cells were capable of helping in this experimental system, we wondered whether the suppressor activity of CD8<sup>+</sup> T cells resided in the CDw60<sup>-</sup> subpopulation. Therefore, a combination of  $5 \times 10^4$  B cells and  $5 \times 10^4$  CD4<sup>+</sup> T cells was mixed either with  $5 \times 10^4$  CD8<sup>+</sup>CDw60<sup>+</sup> or  $5 \times 10^4$  CD8<sup>+</sup>CDw60<sup>-</sup> T cells. As can be seen from Fig. 3, the CDw60<sup>-</sup> subset of the CD8<sup>+</sup> cells almost totally suppressed the IgG production by B cells, whereas the IgG secretion was even increased in the presence of CDw60<sup>+</sup>CD8<sup>+</sup> cells. These data show that the CDw60 marker allows a clear distinction of two functional subsets of CD8<sup>+</sup> T lymphocytes, one containing helper cells, whereas the other harbors cytotoxic and suppressor cells.

**Lymphokine Production by CD8<sup>+</sup> T Cell Subsets.** Since the diverse regulatory activities of T cell subsets are mediated by distinct patterns of secreted lymphokines, we wondered whether the different functions of the CDw60-separated CD8<sup>+</sup> T cells are mirrored by the spectrum of released lymphokines. To this end, CD8<sup>+</sup> T lymphocytes were separated into CDw60<sup>+</sup> and CDw60<sup>-</sup> subsets and stimulated either by PHA or by a combination of PMA and ionophore. For comparison, CD4<sup>+</sup> cells prepared from the same batch of PBL were included. After 1, 2, and 4 d the lymphokines IL-2, IL-4, and IFN- $\gamma$  were determined in the supernatants. Table 1

Cellular composition of cultures



**Figure 3.** Influence of various T cell subsets on the PWM-induced B cell differentiation into IgG-secreting cells.  $5 \times 10^4$  enriched B lymphocytes were cultivated with  $5 \times 10^4$  cells, respectively, of the indicated subsets in the presence of  $50 \mu\text{g/ml}$  of PWM for 7 d. Every cell combination was set up in triplicate. IgG was determined in the supernatants by ELISA. Values shown represent the mean of triplicate samples. SEM was <15%.

shows the values obtained in a representative experiment after 2 d of stimulation. Similar amounts of IL-2 were secreted by both the CDw60<sup>+</sup> and CDw60<sup>-</sup> subpopulation of CD8<sup>+</sup> cells after stimulation with PMA/ionophore. A clear-cut difference was found in the amount of secreted IL-4 that was preferentially produced by CDw60<sup>+</sup> CD8<sup>+</sup> cells after stimulation with PMA/ionophore, whereas the opposite was observed in the production of IFN- $\gamma$ , which was mainly detected in the supernatant of CDw60<sup>-</sup> CD8<sup>+</sup> cells after PHA stimulation.

**Discussion**

In this study, we describe for the first time the phenotypic identification of a CD8<sup>+</sup> Th cell subset. It is based on the surface expression of the CDw60 antigen which has been identified as the terminal 9-O-acetylated disialosyl group pre-

dominantly found on ganglioside GD<sub>3</sub> (8). This antigen was first described as a tumor-associated antigen on melanoma cells (20). More recently, CDw60 mAbs were shown to distinguish functional subsets of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes (9) and to provide costimulatory signals to T cells (15). In the present study, we have extended these investigations. The most striking discovery was the existence of cells in the CD8<sup>+</sup> compartment which are capable of supporting IgG production by B lymphocytes with an efficiency similar to that of CD4<sup>+</sup> T lymphocytes. This helper activity was closely associated with the surface expression of the CDw60 antigen. The presence of Th cells in the CD8<sup>+</sup> lymphocyte subpopulation has been suggested by reports showing that in both humans and mice CD8<sup>+</sup> T lymphocytes were able to produce helper lymphokines such as IL-4 which induce proliferation and differentiation of B cells (3, 21-26). It remains to be shown whether the CD8<sup>+</sup> Th cells are also able to provide help in antigen-specific B cell stimulation and whether CD8-MHC class I interaction is involved in the T cell-B cell collaboration.

The CDw60<sup>-</sup> CD8<sup>+</sup> population comprises both suppressor and cytotoxic T cells. Earlier reports have shown that these two functional subsets can be distinguished by the expression of the CD11b surface antigen (7). When CDw60-separated CD8<sup>+</sup> cells were stained either with a CD11b mAb or with a CD16 mAb, all labeled cells were detected in the CDw60<sup>-</sup> population. These data are consistent with the view that suppression can be mediated by cells with NK activity.

When we separated CD8<sup>+</sup> T cells into the CDw60<sup>+</sup> subset supporting B cell differentiation into IgG-secreting cells and into the antagonistic CDw60<sup>-</sup> suppressor cells, both subsets could be induced to produce IL-2 and IL-4, although the IL-4 secretion by CDw60<sup>+</sup> cells was significantly higher when compared with the CDw60<sup>-</sup> cells. When analyzing T cell clones obtained from patients with

**Table 1.** Lymphokine production by CDw60-separated T cell subsets.

Cell subset	Lymphokine		
	IL-2	IL-4	IFN- $\gamma$
		<i>pg/ml</i>	<i>IU/ml</i>
CDw60 <sup>+</sup> CD8 <sup>+</sup>	505*	127 <sup>†</sup>	5 <sup>†</sup>
CDw60 <sup>-</sup> CD8 <sup>+</sup>	465	35	41
CD4 <sup>+</sup>	559	167	63

\* Secreted IL-2 was determined after 2 d of stimulation with  $1 \mu\text{g/ml}$  of PHA.

<sup>†</sup> Secreted IL-4 and IFN- $\gamma$  were determined after 2 d of stimulation with  $100 \text{ ng/ml}$  of PMA in combination with  $1 \mu\text{g/ml}$  of Ca-ionophore. Values represent the mean of triplicate samples. SEM was <15%.

lepromatous leprosy, Salgame et al. (24) identified CD8<sup>+</sup> cells secreting substantial amounts of IL-4. These clones suppressed the antigen-specific proliferation of CD4<sup>+</sup> T cells, and IL-4 was an indispensable component of their suppressor activity. These findings clearly indicate that the functional capacity of a T cell subpopulation cannot be inferred solely from the pattern of secreted lymphokines. Therefore, inclusion of the phenotype might be of great value in the characterization of functional T cell subsets. Based on the production of IL-4 and IFN- $\gamma$ , the CDw60<sup>+</sup>CD8<sup>+</sup> cells are at first glance reminiscent of the CD4<sup>+</sup> Th2 subset and the CDw60<sup>-</sup>CD8<sup>+</sup> resemble Th1 cells. Membrane expression of glycolipids, which indicates distinct cellular functions, has also been observed in the mouse. There, presence of the gan-

glioside GD1a was restricted to Th2 cells, whereas the ganglioside GD1 $\alpha$  was selectively found on Th1 cells (27).

Using the newly introduced CDw60 surface antigen in combination with already established markers, the following characteristic phenotypes of three functional CD8<sup>+</sup> T cell subsets can be envisaged: antigen-specific cytotoxic cells are CDw60<sup>-</sup>CD11b<sup>-</sup>, cells with suppressor capacity and cells with NK activity are found within the CDw60<sup>-</sup>CD11b<sup>+</sup>CD16<sup>+</sup> population and CD8<sup>+</sup> Th cells are CDw60<sup>+</sup>. A more detailed phenotypic analysis of CD8<sup>+</sup> T cell subsets, particularly the identification of CD8<sup>+</sup> Th cells, may provide new insights into lymphocyte functions in physiological situations and in various disease states such as autoimmune diseases and HIV infection.

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