

Analysis of Lymphocytic Infiltration in Uveal Melanoma

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Among 27 uveal melanomas, five were found to contain tumor infiltrating lymphocytes (TILs). Four had high levels of lymphocytes, and the fifth had comparatively low levels but adequate numbers for comprehensive analysis. The TILs were analyzed by flow cytometry to determine the relative proportions of lymphocyte subsets and markers of lymphocyte activation. The results show the predominance of T-suppressor/cytotoxic lymphocytes and insignificant levels of B-cells present in the infiltrate. The T-suppressor/cytotoxic cells were generally activated to a higher degree than the T-helper cells when assayed for levels of the histocompatibility antigen, HLA-DR. T-helper cells expressed more interleukin (IL-2) receptor (Tac) than T-suppressor/cytotoxic cells. Invest Ophthalmol Vis Sci 31:2106–2110, 1990

Uveal melanomas, like their cutaneous counterpart, are considered to be relatively susceptible to immunologic influences because of reports of spontaneous regression,^{1,2} of the development of vitiligo and halo nevi,³ and of the delayed appearance of metastatic disease, sometimes decades after enucleation.⁴ However, the immunobiologic circumstances are different from the cutaneous disease. Neither the eye nor the orbit are drained by lymphatics so that the primary antigen recognition site is likely to be the reticuloendothelial system in the spleen. In addition, the fact that melanoma is easier to transplant by intraocular inoculation than subcutaneously also indicates specific differences in tumor antigen recognition.⁵ Such intraocular transplantation can be prevented by prior adoptive transfer of humoral or cellular immunity to the recipient.⁶

In approximately 5%–12% of all uveal melanomas, it is possible to identify infiltration of the tumor by lymphocytes,⁷ and many other forms of malignant neoplasms can also be shown to be infiltrated by lymphocytes, consisting mainly of T-cells.^{8–14} Some investigators report the predominance of the T-cytotoxic/suppressor cell subpopulation;^{12,13} others found a majority of T-helper/inducer cells.^{10,11,14} There is, however, no convincing evidence that a more favorable prognosis is associated with lymphocytic infiltration in uveal melanomas,^{7,15} and it remains uncertain whether tumor infiltrating lymphocytes (TILs) play a significant role in tumor immunity. The availability of precisely defined monoclonal-antibody markers and flow-cytometric techniques makes it

possible to do a detailed objective analysis of a large number of lymphocytes in any tumour. We characterized the phenotype of the TILs and studied the expression of the histocompatibility antigen, HLA-DR, and interleukin (IL-2) receptor (Tac), which are markers of activation, on the surface of T-helper and T-suppressor/cytotoxic lymphocytes in ocular melanoma.

Materials and Methods

Preparation of Cells

Tissue was obtained from five choroidal melanomas which were removed either by local resection or by enucleation. Slices were cut from the apical part of the fresh specimen after preliminary examination, and these were transferred to RPMI-1640 medium (Gibco, Grand Island, NY). Spilled cells were teased out using a sterile needle. The cells were then harvested, washed by centrifugation at 1000 rpm for 15 min, counted, and adjusted to 1×10^6 cells/ml. The remaining cell suspensions of TILs and tumor cells were cryopreserved in medium containing 90% fetal calf serum and 10% dimethylsulfoxide and stored in liquid nitrogen until use.

Four of the five patients consented to venipuncture, and peripheral blood lymphocytes were separated on a discontinuous ficoll density gradient.^{16,17}

Preparation of Samples

Cell suspensions of TILs and tumor cells were washed and resuspended in 1 ml of phosphate-buffered saline. Cells ($50 \mu\text{l}$) were incubated on ice with appropriate fluorescein isothiocyanate (FITC)-conjugated leu series monoclonal antibody and phycoerythrin (PE) conjugated antibody (Becton-Dickinson,

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Cowley, Oxford, UK). Simultest (Becton-Dickinson) reagents were also used to determine the levels of T- and B-lymphocytes present and to evaluate the levels of the T-cell subpopulations present. Both TILs and tumor cells were incubated with the antibody at 4°C for 20 min. All samples were maintained in the dark to prevent bleaching. Propidium iodide was added to each tube to a final concentration of 2 µg/ml, and a live/dead discrimination was done. Antigen expres-

sion was then analyzed by flow cytometry using FACScan software (Becton-Dickinson). Ten thousand live gated events were collected for each sample analyzed.

Results

Of 27 tumors analyzed substantial proportions of TILs were found in the tumor suspensions by FACS

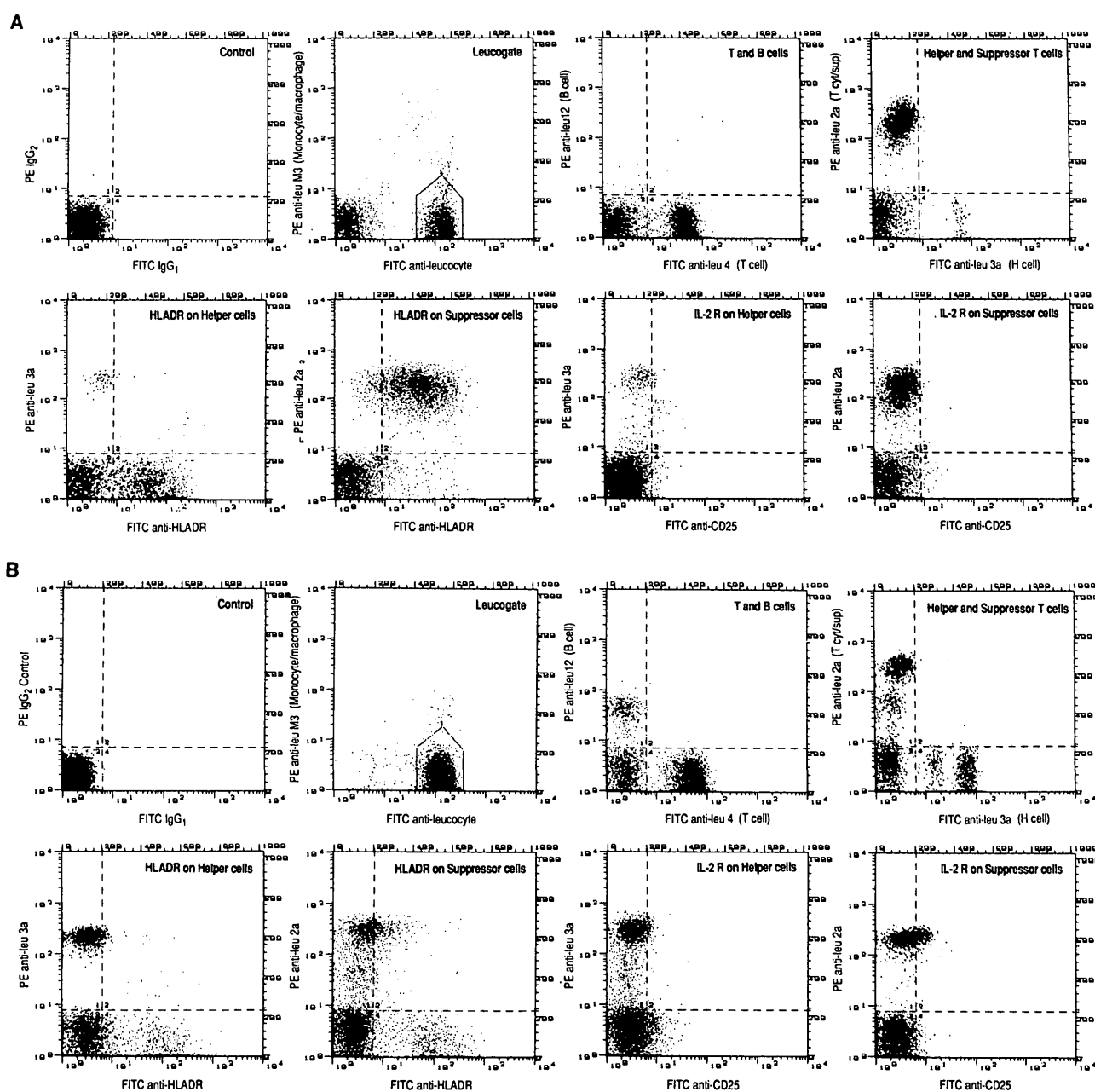


Fig. 1. FACS analysis of (A) tumor infiltrating lymphocytes (TILs) and (B) peripheral blood lymphocytes (PBLs) from a single patient. Lymphocytes were determined using leucogate antibody (FITC-anti-leucocyte and PE anti-Leu-M3, which stains monocytes) with window 1 then being constructed to indicate the position on fluorescence scale compared with nonspecific staining in the control. Subpopulations of T-lymphocytes were analyzed for the presence of HLA-DR and IL-2R (CD25). Activated cells appear in quadrant 2 of the diagram, compared with nonactivated cells in quadrant 1.

using Becton-Dickinson leucogate antibody (anti-leucocyte FITC and anti-leu M3 PE) in four of the five melanomas tested. The fifth tumor, with a comparatively small infiltrate, was also analyzed to investigate the possibility that this lymphocytic infiltrate was a reflection of hemorrhage in the tumor. Significant lymphocytic infiltrate was also identified by routine microscopic techniques. For four of the patients, a venous blood sample was removed immediately before surgery for comparison of blood lymphocytes with TILs. A collection gate was placed around the lymphocytes based on forward-scatter and side-scatter properties to obtain data only from the lymphocytes and not from the larger tumor cells. Samples were then collected with this gate and also with a live/dead discrimination gate set to exclude any cells fluorescing in the far red, ie, cells stained with propidium iodide. These gates confirmed that only live lymphocytes in the tumor were being analyzed.

Figure 1 illustrates the results of an analysis of the lymphocyte subsets and activation markers from both the TILs and the peripheral blood lymphocytes of a single patient. Several differences between the two populations of lymphocytes are apparent. The TILs have few B-lymphocytes in comparison to the blood and a relatively greater number of T-cytotoxic/suppressor cells. The TILs also contain lymphocytes with a greater level of expression of markers of activation as evidenced by the staining with antibodies to HLA-DR and the Tac compartment of the IL-2 receptor. In Figures 2-5, the data obtained in a manner similar to that demonstrated in Figure 1 from all five tumors is presented together with the corresponding data from the peripheral blood lymphocytes of four of the patients. For all patients it can be seen that T-cells predominate in the TILs (Fig. 2). In the

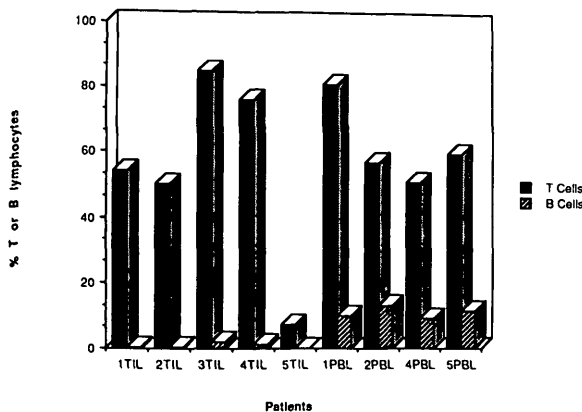


Fig. 2. Levels of T- and B-cells present in TILs and PBLs from five patients with uveal melanoma. The T-cells predominate in both samples.

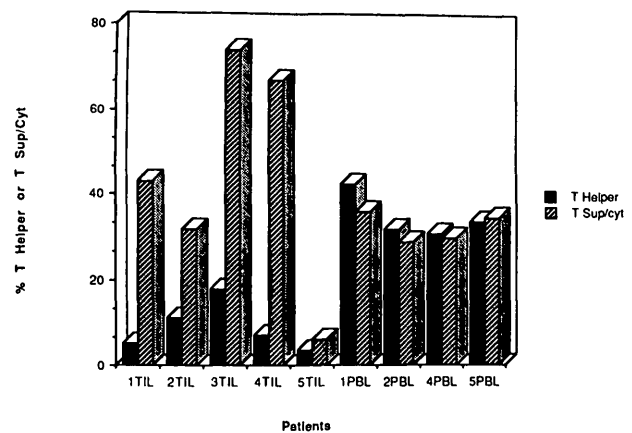


Fig. 3. Levels of T-helper and T-suppressor/cytotoxic cells in TILs and PBLs from five patients with uveal melanoma. Whereas the cytotoxic/suppressor cells predominate in the TILs, the helper cells tend to predominate in the PBLs.

tumors T-cytotoxic/suppressor lymphocytes predominate whereas in the peripheral blood, T-helper cells predominate (Fig. 3).

These individual T-cell subpopulations were stained using the appropriate PE-conjugated anti-leu series antibody. The FITC anti-HLA-DR was then added to determine the presence of this activation marker on the surface of the T-cells. A positive result was then determined by an increase of fluorescence compared with the control. The TILs expressed substantially more HLA-DR than did the peripheral blood lymphocytes even in patient 5 where fewer TILs were present (Fig. 4).

The T-helper cells were consistently more positive for the IL-2 receptor (Tac) than the T-cytotoxic/suppressor subsets (Fig. 5). Levels of IL-2 receptor-positive T-helper cells ranged from 11%–26%. Peripheral blood lymphocytes also expressed highly variable but

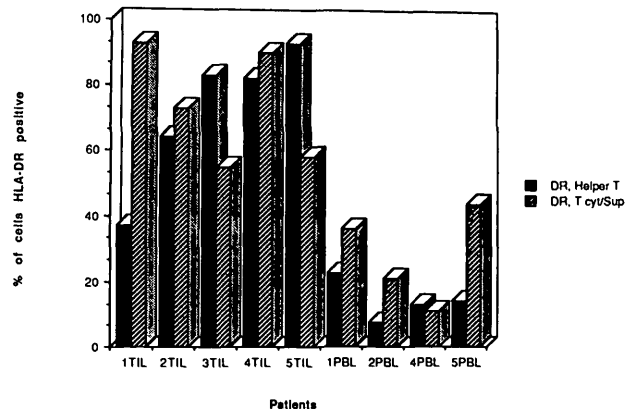


Fig. 4. Levels of HLA-DR on T-helper and T-suppressor/cytotoxic cells in TILs and PBLs from five patients. The TILs, including those of patient 5, express more HLA-DR than the PBLs.

substantial levels of the IL-2 receptor, suggesting that the two markers of activation detected different subpopulations of cells in the samples.

Discussion

The activation of T-helper cells is the beginning of an effective immune response, and the successful completion of antigen presentation by an antigen presenting cell (APC). During the activation process, the surfaces of T-cells acquire HLA-DR antigens and receptors for IL-2.

The activation of T-cells is thought to occur after binding of the T-cells to the APC. T-cells, particularly those of the helper variety, do not recognize free antigen which must be presented together with major histocompatibility complex class 2 products expressed by the APC. The initiation of T-cell proliferation also requires signals between the APC and the lymphocyte via interleukins. This induces the APC to produce IL-1 and, in association with antigen stimulation, induces IL-2 receptors on the T-cells, stimulating T-cells to release IL-2 which causes antigen-activated cells to proliferate.^{8,10,18,19}

This study shows that the TILs of uveal melanomas contain high levels of HLA-DR on the surface of their T-helper cells, possibly indicative of the antigen-presenting capabilities of the T-cells to other T-helper cells, augmenting the immune response.

Two separate IL-2 binding molecules have been reported on human lymphocytes; each of these binds IL-2 alone or together in a heterodimeric receptor complex.^{20,21} These three receptor forms are responsible for three distinct affinities of IL-2 binding: low (Tac alone); medium (p70-p75 alone); and high (the p70+ Tac dimeric complex). A probe for the p70 IL-2

Table 1. Nomenclature of Leu series MAbs

<i>Antibody</i>	<i>Predominant reactivity</i>
IgG ₁ -FITC + IgG _{2a} -PE	Control
Leucogate	Detection of lymphocytes
Anti-Leu 2a	Suppressor/cytotoxic T-lymphocytes
Anti-Leu 3a	Helper T-lymphocytes
Anti-Leu 4	T-lymphocytes
Anti-Leu 12	B-lymphocytes
HLA-DR	Class II MHC antigens
IL-2R	Interleukin-2 receptor (Tac or CD25 component)

receptor is not yet available, but cells lacking Tac cannot express the highest in affinity of these three receptor types. We found that few of the T-cytotoxic/suppressor cells expressed Tac receptor on the surface. It has been suggested that it is possible to expand T-lymphocytes in IL-2 and then readminister these to humans in the hope that the lymphocytes are actually more tumoricidal than lymphokine-activated killer (LAK) cells.²² We reported that the T-cytotoxic/suppressor lymphocytes are low in the receptor for IL-2. If placed into culture with recombinant IL-2, therefore, the T-helper cells might predominate and the T-cytotoxic/suppressor cells population not expand significantly. There is evidence that this phenomenon also occurs in cutaneous melanoma.¹⁰ This may become relevant when considering LAK cells and recombinant IL-2 in the adoptive immunotherapy of patients with metastatic uveal melanoma and other advanced cancers.

Key words: uveal melanoma, lymphocytic infiltration, flow cytometry, immune response

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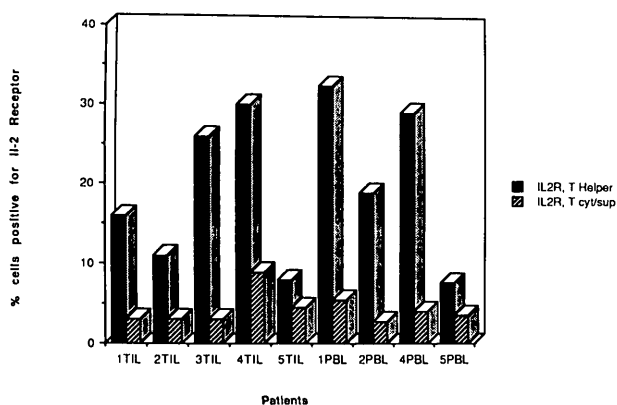


Fig. 5. Levels of IL-2R (CD25) present on T-helper and T-suppressor/cytotoxic cells in TILs and PBLs from five patients with uveal melanoma. In all cases, helper cells have more IL-2R than cytotoxic cells.

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