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First-Generation Monoclonal Antibodies Identifying the Human Leukotriene B_4 Receptor-1

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The leukotriene B_4 receptor (BLTR) is a seven-transmembrane chemoattractant receptor that is important in pro-inflammatory responses. We have produced the first widely applicable monoclonal antibodies against the human BLTR and confirmed the antibody specificity using flow cytometric analysis of three different cell lines stably expressing the recombinant receptor. The antibodies did not cross-react with the recently cloned second LTB_4 receptor, BLTR2, or the Cys LT1 and Cys LT2 receptors. Functional analysis in combination with two-color flow cytometry showed that the BLTR antibodies bind to cells that are activated by LTB_4. The antibodies were shown to recognize BLTR in cell ELISA and immunocytochemistry. Endogenous expression of BLTR in CD15-positive blood leukocytes and in differentiated HL-60 cells was also demonstrated with the antibodies. © 2000 Academic Press

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The leukotriene B_4 (LTB_4) receptor, designated BLTR according to the NC-IUPHAR nomenclature, is a G-protein-coupled chemoattractant receptor whose existence has been predicted since the discovery of the leukotrienes almost three decades ago (1). Although it has been thoroughly examined using classical pharmacological methods, the cDNA encoding the receptor protein was only recently cloned and characterized (2–4). The natural ligand for the receptor, LTB_4, is a dihydroxy acid that is synthesized by the 5-lipoxygenase pathway by transformation of arachidonic acid into leukotriene A_4 which, in turn, is converted by hydration to LTB_4 (1). Upon activation of neutrophils, macrophages, mast cells and certain epithelial cells LTB_4 is released in order to attract additional neutrophils, eosinophils, and monocytes chemotactically (1, 5, 6). Thus, LTB_4 and its receptor play an important role in pro-inflammatory responses. In addition, it has been suggested that BLTR, like certain chemokine receptors and other related inflammatory receptors, can assist CCR5 and CXCR4 in the coreceptor function required for infection of CD4-positive cells with HIV-1 (7).

Using the hybridoma technique we have produced the first monoclonal antibodies with broad applicability identifying the human LTB_4 receptor, and the present report describes the characterization of these antibodies and their application for detection of both the recombinant and endogenous LTB_4 receptor.

MATERIALS AND METHODS

If not otherwise stated, the cell culture medium components came from Life Technologies and the test protocols were performed at room temperature.

Generation of monoclonal antibodies. The monoclonal antibodies recognizing human BLTR were generated by immunizing BALB/c mice with HeLa-derived HF1 cells, which is a reporter cell line designated HeLa-HF1 (8) that has been stably transfected with cDNA (2) to express the full-length BLTR protein. The expression of BLTR on the surface of the transfected cells was demonstrated by specific binding of FITC-labeled LTB_4 (BIOMOL) as well as by functional analysis (8). Cells were detached with 2 mM EDTA (KEBO) in PBS at 80–100% confluency after 3 days of cultivation, and they were then suspended in PBS. Seven million cells in suspension were mixed with an equal volume of MPL/TDM adjuvant (RIBI; Sigma) and injected into each mouse subcutaneously at four sites close to the axillary and epigastric lymph nodes. Injections were repeated once a week during a 4-week period followed by an interval of 6 to 10 weeks. Three days before cell fusion, a final booster with 3×10^7 cells in MPL/TDM adjuvant was given intraperitoneally. Spleen cells taken from immunized animals were fused with SP 2/0 myeloma cells using the polyethylene glycol method according to standard protocols. Supernatants from wells with growing hybridomas were screened using flow cytometric analysis of BLTR-transfected HeLa-HF1 cells and sham-transfected (vector only) cells as negative controls. Cells from positive wells were cloned by repeated limiting dilution.

Cell culture conditions and cell lines. Cells were cultured at 37°C in 7% CO_2 atmosphere in a basal medium consisting, if not otherwise

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stated, of DMEM/Glutamax-I with 10% FBS and 0.5% penicillin-streptomycin. HeLa-HF1 cells—sham-transfected or transfected with either BLTR, BLTR2, or CysLT2—were selected for cDNA expression using 0.75 mg/ml G-418 and 1 μg/ml puromycin (Sigma). The L-VIP reporter cell line (9) was maintained in the presence of 25 μg/ml hygromycin; L-VIP cells transfected with BLTR were supplemented with 0.5 mg/ml G-418. The BLTR-transfected NIH 3T3 cells (7) were grown in the presence of 1 μg/ml puromycin; sham-transfected controls were maintained in the presence of 25 μg/ml hygromycin. HL-60 cells were cultured in RPMI 1640/Glutamax-I supplemented with 10% FBS and 0.5% penicillin–streptomycin. The Cys LT1 receptor was transiently expressed in HEK 293 cells (grown in basal medium) after transfection with the corresponding cDNA using Lipofectamine PLUS (Life Technologies).

Flow cytometry. Eleven incubations were carried out at 4°C in the dark if not otherwise stated. Suspended cells (250,000) from cell lines, or 100 μl of whole blood from healthy donors, were washed once in washing buffer (Dulbecco's PBS w/o Ca^{2+} and Mg^{2+} supplemented with 0.5% BSA [Labora/Chemicon], 5% human serum [Sigma] and 0.05% sodium azide[KEBO]) and resuspended in 50 μl hybridoma supernatant or 50 μl washing buffer containing 0.1 μg isotype control mAbs (IgG1 or IgG2a; Becton–Dickinson). After incubation for 30 min followed by two washes in washing buffer, a secondary incubation was performed for 30 min with phycoerythrin-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin antibody (DAKO), diluted 1:25 in washing buffer. The cells were washed twice and, if they were to be double-stained with FITC-conjugated anti-CD15 (Becton–Dickinson), this antibody was added in 1:10 dilution in washing buffer for 30 min followed by two washes. Red blood cells were lysed by adding 2 ml FACS lysing solution (Becton–Dickinson) diluted 1:10 in deionized water and incubated for 10 min at room temperature prior to a final wash. Besides labeling of the surface receptors with mAbs, expressed BLTR was also detected by binding with FITC-labeled ligand, LTB_4. Binding experiments using 33 nM LTB_4-FITC and HeLa-HF1 cells were performed using the same method as for the antibodies, and binding was blocked using unlabeled LTB_4 (Sigma) at 10 μM to control for any nonspecific binding. The cells were fixed in 0.5% paraformaldehyde and stored at 4°C until analysis (sample size: 10,000 cells) in a Becton–Dickinson FACS Calibur flow cytometer using the CellQuest software. For cultured cell lines a major population of viable single cells was selected and analyzed following forward and side scatter gating; with whole blood cells, gating was performed to study the granulocyte population (other cell types were not examined).

Immunocytochemistry. HeLa-HF1/BLTR, HeLa-HF1/sham, L-VIP/BLTR and L-VIP cells were seeded onto 12-well multi test slides (Labora/Chemicon) and cultured until confluency. The slides were washed three times with PBS followed by blocking for 1 h at 4°C with PBS containing 0.5% BSA. The primary antibody, 10 μl per well of hybridoma supernatant or 0.1 μg/10 μl of isotype control, was incubated for 1 h at 4°C followed by three washes in PBS/BSA. For detection of bound primary antibody, goat anti-mouse IgG antibody conjugated to FITC (Jackson ImmunoResearch) diluted 1:20 in PBS/BSA was then added and incubated for 1 h at 4°C, followed by washing three times in PBS/BSA and once in deionized water. Human blood leukocytes were labeled according to the flow cytometry protocol and the cells were centrifuged onto microscope slides using a Cytospin3 (Shandon) centrifuge. The slides were counterstained with 4′-6-diamidino-2-phelylindole (DAPI; Sigma) and mounted under coverslips with glycerol. The slides were photographed using a fluorescence microscope equipped with a Hamamatsu CCD camera, and the images colored using Openlab 2.2.2 (Improvision).

Cell ELISA. The antigens expressed on the cell surface were also detected using a modification of cell ELISA (10). HeLa-HF1/BLTR and HeLa-HF1/sham cells were seeded into 96-well plates three days prior to analysis. All dilutions and washes were made in cell wash medium (DMEM supplemented with 5% FBS and 50 mM Hepes). The cells were washed once before addition of 100 μl hybridoma supernatant and incubated for 2 h at 4°C before washing three times and addition of 100 μl goat anti-mouse IgG + IgM antibody conjugated to horseradish peroxidase (HRP; Jackson ImmunoResearch) diluted 1:1,000. The secondary antibody was incubated together with the cells for 1h at 4°C followed by four washes in cell wash medium and a final wash in PBS with Ca^{2+} and Mg^{2+}. HRP was detected using the substrate, *o*-phenylenediamine (OPD; Sigma), in citric acid buffer and 0.04% H_2O_2. The reaction was stopped with 1 M HCl and the result analyzed at 490 and 650 nm wavelength in a micro-plate reader.

Reporter cell assay. The HeLa-HF1 cells stably expressing BLTR were seeded into 6-well plates at a density of 3×10^5 cells/well, and when confluent stimulated with LTB_4 (100 μM ethanol solution) at a final concentration of 1 μM. Three controls were used: HeLa-HF1/BLTR cells and HeLa-HF1/sham cells incubated in the presence of ethanol (1:100) and HeLa-HF1/sham cells stimulated with LTB_4. After 16 h of stimulation the cells were detached using 2 mM EDTA in PBS and stained with anti-BLTR antibodies for two-color flow cytometry.

RESULTS AND DISCUSSION

Flow cytometric screening of hybridoma supernatants from two cell fusion experiments resulted in a total of five antibodies. The two antibodies (designated 7B1 and 14F11) that gave the strongest signals were chosen for further characterization and are reported in the present study. They constitute the first widely applicable monoclonal antibodies identifying the LTB_4-receptor, BLTR (2–4), and are suitable for, e.g., flow cytometry, cell ELISA and fluorescence cytochemistry. The antibodies were obtained following immunization of mice with cells stably expressing the recombinant human (BLTR) receptor, which is the way antibodies generated against other G-protein-coupled receptors have often been produced (11, 12). A previously developed BLTR antibody, raised in mice immunized with a synthetic N-terminal receptor peptide and used in a preliminary fluorescence cytochemical study (4), has not been further pursued because of its limited applicability.

The receptor expression in the BLTR-transfected HeLa-HF1 cells which were used for the immunization had previously been confirmed by monitoring intracellular signaling following activation with LTB_4 (8). Proper receptor expression on the cell surface was further confirmed prior to the actual immunization by binding of FITC-labeled LTB_4 followed by flow cytometry (Fig. 1). The specificity of the binding was controlled by blocking with non-labeled LTB_4; sham-transfected cells did not bind LTB_4-FITC.

Following fusion of the spleen cells from immunized mice with SP 2/0 myeloma cells the supernatants from growing hybridomas were screened by flow cytometry, which detected a high density of receptors in BLTR-transfected HeLa-HF1 cells (Fig. 2A). The comparatively high fluorescence signal obtained with these cells was probably due to a high degree of receptor expression. This may be related to the efficient method

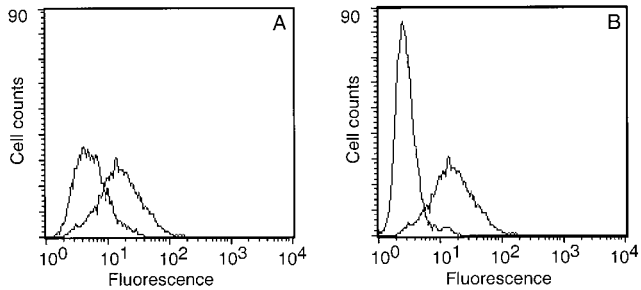

FIG. 1. Flow cytometry using HeLa-HF1 cells exposed to FITC-labeled LTB_4, alone or in combination with unlabeled LTB_4. (A) LTB_4-FITC binds to HeLa-HF1/BLTR cells, and this binding curve is left-shifted due to the blocking by nonlabeled LTB_4. (B) Binding of FITC-labeled LTB_4 to cells expressing BLTR (right curve) and background binding in sham-transfected cells (left curve).

for clonal selection built into the system when it is being used as reporter for receptor activation (8). Sham-transfected cells displayed only background binding. It was also important to make sure that the antibodies detected the recombinant receptor when it was expressed in other cell lines. Thus, it was found that BLTR expressed in two mouse fibroblast cell lines, L-VIP (Fig. 2B) and NIH 3T3 (Fig. 2C), were also efficiently detected by the antibodies. Sham-transfected control cells not expressing BLTR were not recognized by the antibodies (Figs. 2A–2C).

Functional experiments illustrated in Fig. 3 were carried out using HeLa-HF1 reporter cells stably expressing BLTR (8). Stimulation with LTB_4 induced activation of BLTR in the cells, further confirming the expression of functional receptors (Fig. 3B). It was

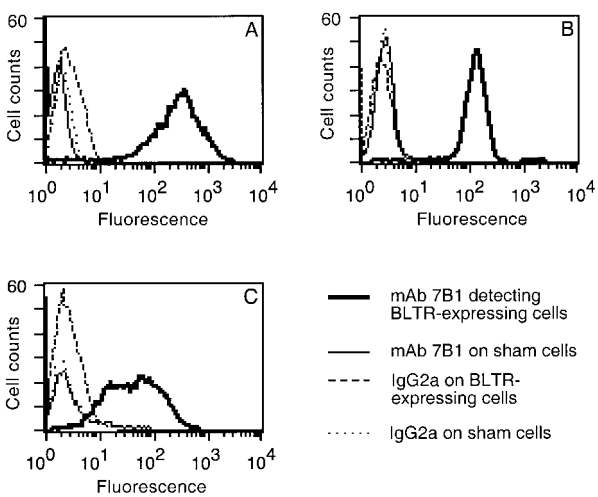

FIG. 2. Detection of BLTR by mAb 7B1 in three cell lines, HeLa-HF1 (A), L-VIP (B), and NIH 3T3 (C) expressing recombinant BLTR. The antibody binding is high and distinct from the corresponding isotype control (IgG2a), and it reflects a varying degree of receptor expression, which is highest in the HeLa-HF1 cells. The binding to sham-transfected controls is always close to the respective isotype control (IgG2a).

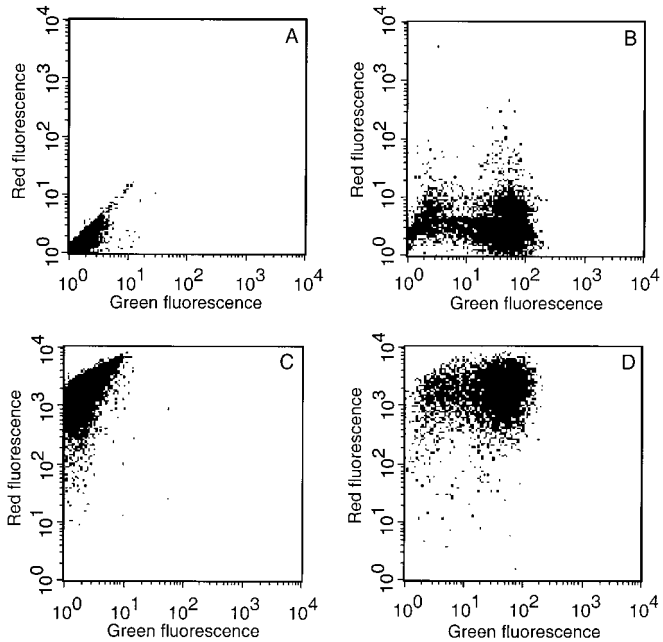

FIG. 3. Flow cytometry (two-color analysis with green and red fluorescence) of BLTR-transfected HeLa-HF1 reporter cells. In A, the cells are not stimulated and not mAb-labeled (but exposed to ethanol, the solvent for LTB_4). Upon stimulation with LTB_4 (B) most cells exhibit a green fluorescence due to activation of the BLTR-linked reporter. In C, nonstimulated cells are labeled with mAb 7B1, indicated by the red phycoerythrin fluorescence. Antibody 7B1 labeling following LTB_4 stimulation (D) showing that the antibody binds to all cells, most of which are activated by LTB_4. Similar results were obtained with mAB 14F11.

possible to identify these stimulated cells by flow cytometric measurements of the green fluorescent protein produced by the activated reporter construct (8). Staining of the cells with either of the monoclonal antibodies against BLTR resulted in a phycoerythrin-induced red fluorescence signal from essentially all cells (Fig. 3C). Staining of LTB_4-stimulated, green-fluorescent cells with the red-fluorescent antibody showed that all cells expressed BLTR and that most of them were functionally active (Fig. 3D). This would indicate that the antibodies also bind to inactive receptors. The proportion of these cells varied in different experiments. As noted also previously (8) sham-transfected reporter cells, whether exposed to LTB_4 or ethanol solvent, were not activated (data not shown).

It has recently been shown that LTB_4 activates a second seven-transmembrane receptor which has been named BLTR2 (13–16). The two LTB_4 receptors belong to a group of cloned leukotriene receptors which also includes the CysLT1 and CysLT2 receptors. In flow cytometric analysis (data not shown) neither of the antibodies detected any of the leukotriene receptors other than BLTR (which, logically, should be designated BLTR1). Receptor expression in these cells was

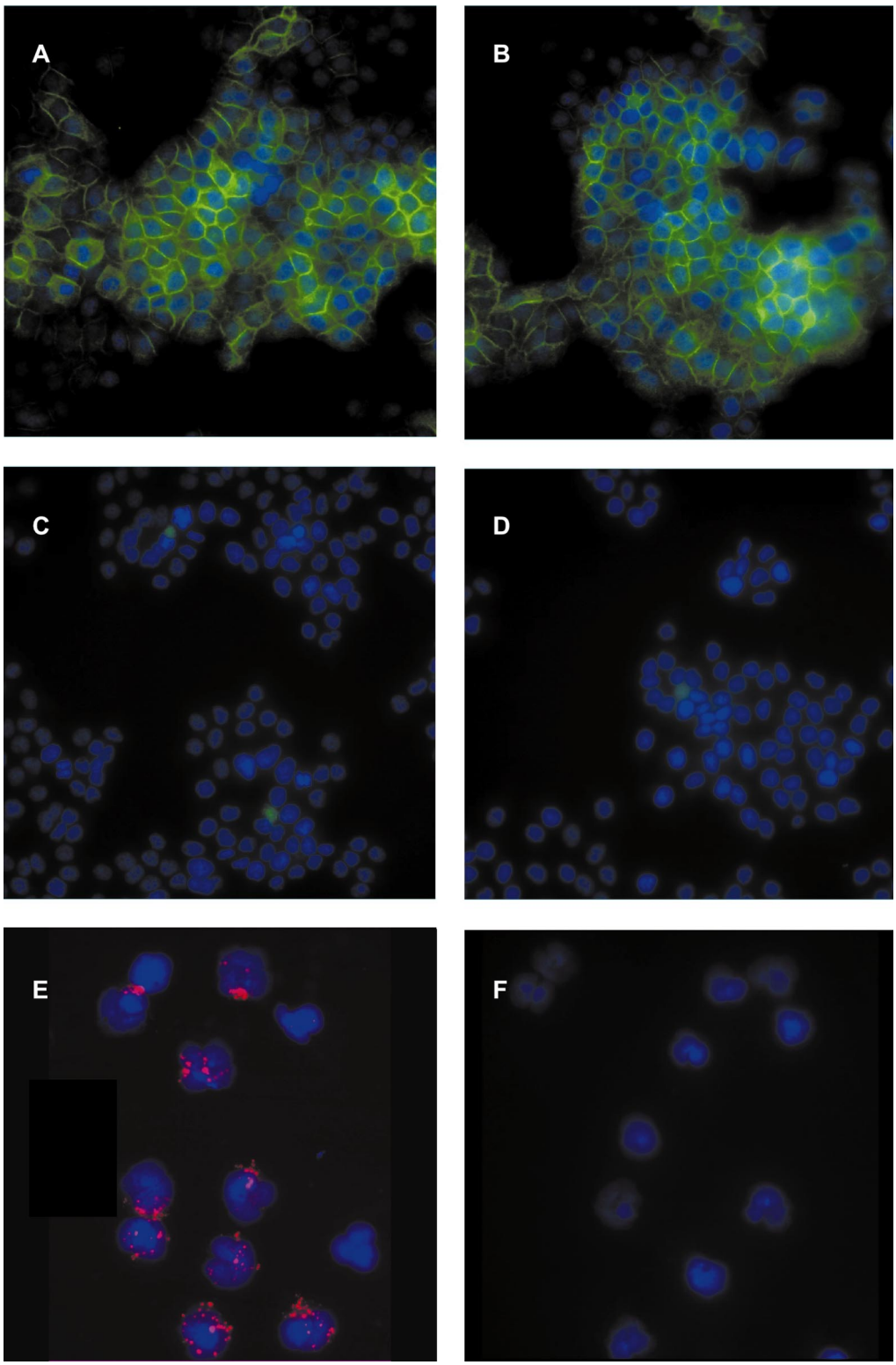

FIG. 4. Immunocytochemical demonstration of the distribution of BLTR expressed in transfected HeLa-HF1 cells (FITC-labeling) and blood leukocytes (phycoerythrin-labeling). Panel A visualizes mAb 7B1, panel B mAb 14F11. (C) Absence of mAb 7B1 antibody binding to sham-transfected cells, and (D) absence of binding of the isotype control, IgG2a, to BLTR-expressing cells. The different pattern of binding of mAb 7B1 to blood leukocytes is illustrated in E along with the negative binding of the isotype control, IgG2a (F). The blue fluorescence is due to the counterstaining with DAPI. Original magnifications: 400× (A–D); 500× (E, F).

confirmed in functional tests with the respective natural ligand (data not shown).

To determine whether or not the antibodies could be used in other immunochemical techniques they were tested using immunocytochemistry and cell ELISA. Fluorescence microscopy of BLTR-transfected HeLa-HF1 cells showed the immunoreactivity to be localized to the cell surface corresponding to the way membrane receptors would be distributed throughout the circumference of the cells (Figs. 4A and 4B). The fluorescence yield was similar with either antibody. Sham-transfected cells (illustrated for mAb 7B1) displayed only background fluorescence (Fig. 4C), as did the cells (BLTR-expressing cells illustrated) exposed to the isotype control, IgG2a (Fig. 4D). Both antibodies produced a strong signal also in the human leukocytes isolated from whole blood. The fluorescence was located in granular patches scattered at the cell surface, as illustrated for mAb 7B1 in Fig. 4E. Only background fluorescence was seen with the isotype control (Fig. 4F).

ELISA measurements of HeLa-HF1 cells produced a mean value for relative absorbance of 0.08 ± 0.04 (SEM; $n = 3$) in sham-transfected cells for both the 7B1 and 14F11 antibodies. For the BLTR-expressing cells the corresponding values were significantly higher, 1.15 ± 0.20 and 1.20 ± 0.22, respectively ($n = 3$). Cell ELISA would thus be a useful alternative to flow cytometry when screening for potential antibodies against cell surface markers.

The ability of the monoclonal antibodies to detect endogenous BLTR by flow cytometry was exemplified by the use of whole blood and HL-60 cells. In blood, the granulocyte population, known to express BLTR (5), was identified using forward and side scatter gates and, in addition, the cells were labeled with anti-CD15 antibodies. As shown in Fig. 5A, the labeling with antibody 7B1 (like 14F11, not illustrated) suggests the presence of numerous leukotriene B_4 receptors on the CD15-positive blood cells. The lower anti-CD15 fluorescence may reflect a smaller CD15 population compared to BLTR, and the fact that the CD15 antibody used was directly conjugated to FITC. The presence of BLTR in human granulocytes has recently been demonstrated using (polyclonal) antiserum raised in rabbits (17).

The human leukemia cell line, HL-60, can be differentiated into neutrophil-like cells following exposure of DMSO (18). Binding of LTB_4 to the differentiated HL-60 cells is increased which, in fact, has been used to facilitate the cloning of BLTR (3). Using flow cytometry the 14F11 antibody readily identified a population among the differentiated HL-60 cells that was distinct from nondifferentiated cells as well as from the respective isotype control (Fig. 5B). On the other hand, it was not possible to distinguish between the binding of the other antibody, 7B1, and the isotype control, IgG2a,

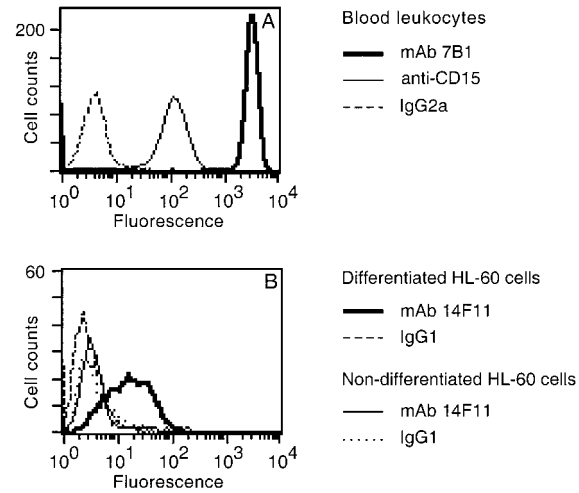

FIG. 5. Anti-BLTR binding to endogenous receptors. Flow cytometry performed with whole blood (A) gated for granulocytes and double-stained with anti-BLTR (7B1), detected with phycoerythrin-conjugated (red) secondary antibody, and FITC-conjugated (green) anti-CD15. The two fluorescence curves, placed in the same diagram, show that all cells in the gated sample bind both mAb 7B1 and anti-CD15, and that the binding is distinct from that of the isotype control (IgG2A). B shows that differentiation of HL-60 cells generates a population which binds mAb 14F11 distinct from the binding of isotype control. The latter is overlapping the binding of 14F11 and its isotype control in nondifferentiated cells.

because HL-60 cells were found to express Fc-receptors for IgG2a mouse immunoglobulin (data not shown).

In conclusion, the present study describes the production and characterization of the first monoclonal antibodies raised against the originally cloned human LTB_4 receptor, BLTR (2–4). The two antibodies described, 7B1 and 14F11, were found to identify both the recombinant and the endogenous receptor with high efficiency and specificity, and they were shown to be broadly applicable for flow cytometry, cell ELISA, and immunocytochemistry. The antibodies should be very useful in characterizing and mapping the distribution of this major leukotriene B_4 receptor, and they will help to distinguish between the different types of leukotriene B_4 receptors (13–16) which has not been possible earlier using biochemical and pharmacological approaches.

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