Flow Cytometric Detection of Receptors for Interleukin-6 on Bone Marrow and Peripheral Blood Cells of Humans and Rhesus Monkeys

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INTERLEUKIN-6 (IL-6) is involved in the regulation of immune responses, acute-phase reactions, and hematopoiesis. Detailed information about the distribution of cell surface receptors for IL-6 is essential to elucidate the target cell specificity of IL-6 and to explain the mechanisms via which IL-6 mediates its multiple biologic activities. Consistent with the pleiotropic activities of this growth factor, IL-6 receptors have been identified on many cell types in different tissues. Binding studies with radiolabeled IL-6, with specific cDNA probes, and with antireceptor antibodies have indicated that the IL-6 receptor is present on hepatocytes, normal bone marrow (BM), and peripheral blood cells and also on leukemic blast cells from patients with acute leukemia.

High-affinity binding sites have been identified on purified populations of activated B lymphocytes and resting T lymphocytes of human as well as rodent origin. However, in other studies, human lymphocytes were found to express only low levels of the IL-6 receptor, in contrast to monocytes and macrophages which expressed the IL-6 receptor abundantly. These discrepancies could reflect variations in purity of the cell populations used and/or differences in the analytical approaches used to examine receptor expression. Binding studies with radiolabeled ligands can provide information about binding kinetics, receptor affinity, and average receptor expression per cell but do not provide insight into the distribution of receptors on different cell types in heterogeneous cell populations unless these cells are purified first. For this reason, very little information is available on the expression of IL-6 receptors on the relatively rare hematopoietic stem cells and immature progenitor cells in BM. Antibodies against the 80-kDa α subunit of the IL-6 receptor can be used to examine receptor distribution on subsets of cells in heterogeneous cell populations by means of multiparameter flow cytometry. However, the expression of the α subunit may not be indicative of the functional high-affinity IL-6 receptors which consist of heterodimers of the α subunit and a 130-kDa, non–IL-6 binding β subunit. In addition, the β subunit is not specific for the high-affinity IL-6 receptor only, because it is also an essential component of the receptors for leukemia inhibitory factor and oncostatin M. To circumvent the disadvantages associated with the use of subunit-specific reagents, we developed a flow cytometric method using biotinylated, functionally active IL-6 to examine the distribution of IL-6 receptors on peripheral blood and BM cells in humans and rhesus monkeys. This highly specific and sensitive approach was used to compare IL-6 receptor expression with the light-scatter properties and cell surface antigen expression of specific subsets of cells in peripheral blood. The same approach was also applied to examine IL-6 receptor distribution on immature hematopoietic progenitor cells as well as more mature cell types in human and rhesus monkey BM to investigate the mechanism of hematopoietic stimulation mediated by IL-6.

MATERIALS AND METHODS

Biotin-labeling of IL-6. Human glycosylated recombinant IL-6, produced in Chinese hamster ovary cells expressing the human IL-6 cDNA was provided by Ares-Serono (Geneva, Switzerland). Before biotin-labeling, IL-6 was diluted to a concentration of 0.45 mg/mL using 0.1 mol/L sodium bicarbonate containing 0.02% (vol/vol) Tween-20 and passed over a column of Sephadex G25 (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer to remove possible buffer components containing amino groups, which interfere with...
biotin (NHS-biotin; Pierce, Rockford IL) in dimethyl sulfoxide (DMSO) was then added to a 60-μL aliquot of the IL-6 to yield a 100-fold molar ratio of biotin to IL-6. The optimal NHS-biotin/IL-6 ratio was determined by titration. After 3 hours of incubation at 20°C, the biotin-labeling was stopped by adding 1 μL 2 mol/L TRIS pH 8.0. Biotin–IL-6 was then separated from free reagent by size exclusion chromatography using a 1-mL column of Sephadex G25 equilibrated in phosphate-buffered saline (PBS) containing 0.02% (vol/vol) Tween-20. The eluate was diluted five-fold with PBS plus 0.02% Tween, aliquoted, and stored at −70°C. Recovery of biologic activity, as measured by [3H]thymidine incorporation into IL-6-dependent B9 hybridoma cells,12 was 80%. The biotinylation efficiency was greater than 99%, as determined by adsorption to agarose beads combined with fluorostaining using a 1-mL column of Sephadex G25 equilibrated in phosphate-buffered saline (PBS) containing 0.02% (vol/vol) Tween-20. The eluate was diluted five-fold with PBS plus 0.02% Tween, aliquoted, and stored at −70°C. Recovery of biologic activity, as measured by [3H]thymidine incorporation into IL-6-dependent B9 hybridoma cells,12 was 80%. The biotinylation efficiency was greater than 99%, as determined by adsorption to agarose beads coated with streptavidin (Sigma, St Louis, MO) and measuring the biologic activity left in the supernatant. Preparation of peripheral blood and bone marrow cells. Heparinized peripheral blood was obtained from healthy human volunteers, after informed consent, and from young adult rhesus monkeys (Macaca mulatta), 3 to 4 years of age. Normal BM samples were obtained from allogeneic BM transplant donors and from rhesus monkeys. Red blood cells were lysed by incubating the cells for 15 minutes at 0°C with 10 mmol/L potassium bicarbonate, 0.1 mmol/L L EDTA, and 155 mmol/L ammonium chloride buffer, pH 7.4. Mononuclear cell preparations were prepared by density centrifugation using Ficoll (Organon Teknika, Durham NC). In some experiments, BM cells were further enriched for progenitor cells by centrifugation over a discontinuous BSA gradient, followed by removal of T lymphocytes by E-rosetting sedimentation, as described.13,14 The resulting cell populations were depleted of CD11b+ cells and enriched for CD34+ cells by immunomagnetic separation. In short, cells were adsorbed to protein A–conjugated immunomagnetic beads (Dynal; Oslo, Norway) to which an anti-CD11b monoclonal antibody (MoAb) had been noncovalently bound, followed by immunomagnetic separation and washing. The purity of CD34+ cells was 95%, as determined by counting of rosettes of cell-bound immunomagnetic beads. Cells bound to the anti-CD34/protein A beads were recovered by competitive elution with excess bovine Ig. Cells were incubated in HHBS containing 2% FCS during 1 hour at 37°C before incubation with biotin–IL-6 to permit release of endogenous IL-6 from IL-6 receptors and to promote expression of unoccupied receptors. Incubation of cells with biotin–IL-6 and antibodies against cell surface antigens. To detect IL-6 receptor expression, 1 × 10⁶ cells in a volume of 100 μL HHBS containing 2% FCS and 0.05% (wt/vol) sodium azide (HFN) were incubated with biotin–IL-6. The optimal biotin–IL-6 concentration, as determined by titration and used in most experiments, was 2.5 nmol/L. Nonspecific binding of biotin–IL-6 was determined by competitive inhibition with a 100-fold molar excess of unconjugated IL-6. Staining with biotin–IL-6 was performed at 4°C during 2 to 2.5 hours in the presence of sodium azide because optimal binding of IL-6 is achieved under these conditions without significant internalization of IL-6/CD22 complexes.15 After incubation, the cells were washed twice with HFN buffer and resuspended in 100 μL HFN buffer containing R-phycocerythin-conjugated streptavidin (SA-RPE; Molecular Probes, Eugene, OR) at a concentration of 7 μg/mL. The cells were then incubated for 45 minutes at 4°C, washed again, and analyzed on a flow cytometer. For analysis of IL-6 receptor expression on lymphocyte subsets using two-color flow cytometry, aliquots of cells were incubated with fluorescein-labeled MoAbs against B cells, T cells, and NK cells; ie, CD19 (Leu-12), CD4 (Leu-3a), CD8 (Leu-2a), and CD56 (Leu-19), simultaneously with the incubation with SA-RPE. MoAbs were used at concentrations recommended by the manufacturer (Becton Dickinson, Mountain View, CA) and 2% (vol/vol) normal human serum or normal rhesus monkey serum was added as a source of irrelevant immunoglobulin to prevent nonspecific antibody interactions. Analysis of IL-6 receptor expression on immature human hematopoietic cells was performed by two-color flow cytometry on light-density BM cells depleted of T lymphocytes and CD11b-expressing cells using FITC-labeled anti-CD34 MoAb 8G1216 at a concentration of 10 μg/mL.

Flow cytometry. Receptor expression was analyzed by immunofluorescence using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). For two-color experiments, spillover of green fluorescence into the red fluorescence detector was electronically compensated to background levels using cells stained with the respective fluorescein-conjugated antibodies only. List mode data were collected for 10,000 events either ungated or in an electronic gate for cells displaying specific light-scatter properties to permit analysis of receptor expression on small subsets of cells. The data were analyzed using the FACScan and Consort 30 software (Becton Dickinson). Mean fluorescence intensities in biotin–IL-6 titration experiments were calculated in light scatter and fluorescence gates for IL-6 receptor–positive lymphocytes, granulocytes, and monocytes. The fluorescence intensity of cells incubated with a 100-fold excess of unlabeled IL-6, as control for nonspecific binding of biotin–IL-6, was subtracted to yield specific fluorescence.

RESULTS
Detection of IL-6 receptors on peripheral blood cells. To examine IL-6 receptor distribution on normal blood cell subsets, nucleated peripheral blood cells from a rhesus monkey were stained with biotin–IL-6 and SA-RPE and analyzed by flow cytometry. Fluorescence results were then analyzed on granulocytes, monocytes, and lymphocytes, which were identified by their characteristic light-scatter properties (Fig 1A). As illustrated in Fig 1B, most granulocytes expressed IL-6 receptors, as demonstrated by the specific staining by biotin–IL-6 of almost the whole cell population in the granulocyte light-scatter window (Fig 1B). Monocytes also expressed significant levels of the IL-6 receptor (Fig 1C), whereas IL-6 receptor expression could be detected on a large subset of cells in the lymphocyte window as well (Fig 1D).

To determine whether the IL-6 binding sites on granulocytes, monocytes, and lymphocytes represented high- or low-affinity IL-6 receptors or both, normal peripheral blood cells were incubated with biotin–IL-6 in a range of 0.01 nmol/L to 7.5 nmol/L. The mean specific fluorescence intensity of the receptor-positive cells within the lymphocyte, granulocyte, and monocyte light-scatter windows was then measured for each biotin–IL-6 concentration. IL-6 receptor–positive rhesus monkey lymphocytes were detectable at the lowest biotin–IL-6 concentration tested, 0.01 nmol/L, whereas maximal specific binding was achieved at a biotin–IL-6 concentration of approximately 0.83 nmol/L (Fig 2A). IL-6 receptors on monocytes and granulocytes were barely detectable after staining with biotin–IL-6 at a concentration of 0.01, 0.03, or 0.09 nmol/L, whereas saturation of binding was not achieved below 7.5 nmol/L, the highest biotin–IL-6 concentration used (Fig 2A). The requirement for relatively high biotin–IL-6 concentrations to saturate IL-6 receptors on monocytes and granulocytes as compared with lymphocytes indicated that
IL-6 receptor expression on blood cell subsets. (A) Dot plot of forward-angle versus right-angle light-scatter properties of nucleated rhesus monkey peripheral blood cells. The rectangular boxes indicate the electronic windows used for analysis of fluorescence data for granulocytes, lymphocytes, and monocytes. (B through D) IL-6 receptor expression on the blood cell subsets as defined in (A): cells stained with biotin–IL-6 (2.5 nmol/L) and SA-RPE (—); nonspecific binding of biotin–IL-6, as determined in the presence of a 100-fold mmol/L excess of unlabeled IL-6 (——); cells incubated with SA-RPE only (……). Note that nonspecific binding of biotin–IL-6 was absent on granulocytes and lymphocytes and low on monocytes.

most IL-6 receptors on these cells bind IL-6 with low affinity, whereas lymphocytes appear to display predominantly high-affinity IL-6 receptors. As shown in Fig 2B, IL-6 receptors were also detectable on human peripheral blood cells. Receptor expression on lymphocytes and monocytes appeared to be similar in both species, but human granulocytes generally expressed lower IL-6 receptor levels than their rhesus monkey counterparts.

IL-6 receptor expression on lymphocyte subsets. To further examine the distribution of IL-6 receptors on lymphocytes, peripheral blood mononuclear cells were stained with biotin–IL-6 and SA-RPE and counterstained with fluorescein-conjugated antibodies against cell surface antigens expressed on lymphocyte-subsets. As shown in Fig 3A and B, IL-6 receptors were readily detectable on CD19⁺ human peripheral blood lymphocytes but could not be detected on CD19⁻ lymphocytes, indicating that B lymphocytes do not express detectable levels of the IL-6 receptor. Most IL-6 receptor-expressing cells were found in the CD4⁺ subset, demonstrating that helper T lymphocytes express the IL-6 receptor (Fig 3C). IL-6 receptors were also detectable on a small subset of CD4⁻ lymphocytes but at lower levels than found on CD4⁺ cells (Fig 3D). The frequency of the IL-6 receptor-positive/CD4⁻ lymphocytes (12% ± 4%; average ± SD for five donors) was similar to the frequency of CD8⁺ lymphocytes (15% ± 5%), suggesting that both populations were identical. As shown in
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Fig 2. Detection of IL-6 receptor expression on rhesus monkey (A) and human (B) blood cell subsets. Nucleated peripheral blood cells were stained with biotin-IL-6 at the indicated concentrations and were analyzed by flow cytometry after staining with SA-RPE. Mean fluorescence intensities were measured for IL-6 receptor-positive cells; ie, a subset of lymphocytes, all monocytes, and all granulocytes (Fig 1). The mean specific fluorescence intensities were calculated by subtracting the mean fluorescence of cells that had been incubated with biotin-IL-6 in the presence of a 100-fold excess of unlabeled IL-6 as a control for nonspecific fluorescence.

Fig 3E, CD8+ lymphocytes indeed expressed IL-6 receptors but at relatively low levels as compared with CD8- cells (Fig 3F). As estimated from the relative specific fluorescence intensities, the difference in IL-6 receptor levels between the CD4+/CD8- and the CD4-/CD8+ lymphocyte subsets was approximately fourfold. In titration experiments, IL-6 receptor expression was detectable on both CD4+ and CD8+ cells using biotin-IL-6 concentrations as low as 10 pmol/L, demonstrating that both lymphocyte subsets express high-affinity sites (data not shown). IL-6 receptor expression was not detectable on cells expressing the CD56 antigen, indicating that IL-6 receptors are not expressed at detectable levels on natural killer (NK) cells (Fig 3G and H).

Expression of IL-6 receptors in normal bone marrow. To examine IL-6 receptor distribution on hematopoietic cells, Ficoll-separated BM cells were incubated with biotin-IL-6 and SA-RPE and analyzed by flow cytometry. Most granulocyte precursors in the light-density fraction of normal rhesus monkey BM (G window in Fig 4A) expressed IL-6 receptors (Fig 4B). IL-6 receptor levels were comparable to those observed on mature granulocytes in peripheral blood (Fig 1B). The majority of erythroid precursors and lymphoid cells (E + L window in Fig 4A) displayed background fluorescence only (Fig 4C). A small subset of IL-6 receptor-positive cells could be distinguished in the E + L light-scatter window, but these cells probably represented mature, peripheral blood-derived T lymphocytes because they were not detectable in T-cell-depleted BM preparations (data not shown). As shown in Fig 4D, a small, but distinct, subset of IL-6 receptor-positive cells could be detected in the light-scatter window containing blast cells (B window in Fig 4A). These IL-6 receptor-positive blast cells represented 2.4% ± 0.9% of the mononuclear cell fraction (29.8% ± 8.9% of the blast cells; average ± SD of nine separate experiments) and were still detectable after T-cell depletion. To examine IL-6 receptor expression on immature hematopoietic cells, progenitor cell-enriched BM cells, ie, depleted of mature cells by density centrifugation, removal of T lymphocytes, and of CD11b+ cells, were further
enriched for cells expressing the CD34 antigen using immunomagnetic beads coated with anti-CD34 MoAb ICH3. As illustrated in Fig 5A, IL-6 receptors were detectable on a subset (~34%) of the positively selected cells, demonstrating that CD34+, immature hemopoietic rhesus monkey cells express IL-6 receptors. IL-6 receptors were not detectable on cells that remained unbound to the anti-CD34 beads, ie, CD34- cells (data not shown), indicating that IL-6 receptor expression on the CD34-enriched cells was not attributable to contaminating CD34+ cells.

IL-6 receptor expression was also examined on human BM cells. Expression levels on human granulocyte precursors were lower than on their rhesus monkey counterparts, as was observed earlier for mature granulocytes in peripheral blood (Fig 2B). IL-6 receptor expression on CD34+ human BM cells was examined by dual-color flow cytometric analysis of biotin-labeled IL-3-stained cells that were counterstained with FITC-conjugated anti-CD34 MoAb 8G12. As illustrated in Fig 5B, IL-6 receptors were detectable on CD34+ human BM cells, but IL-6 receptor levels were twofold to threefold lower as compared with CD34+ cells in rhesus monkey BM (Fig 5A).

**DISCUSSION**

In this study, we have used biologically active biotin-labeled IL-6 to examine the distribution of the IL-6 receptor on phenotypically defined subsets of human and rhesus monkey blood and BM cells. A major advantage of this flow cytometric approach is the possibility to detect receptor expression on phenotypically characterized subsets of cells in unfractionated peripheral blood and BM by relating receptor expression to light-scatter and cell surface antigen expression characteristics. An additional advantage is that receptor expression is detected via binding of the labeled ligand itself, thus providing a more reliable estimate for the capacity of cells to bind IL-6 than can be obtained using probes specific for individual receptor subunits.

Parallel analysis of IL-6 receptor expression on human and rhesus monkey blood cells led to the observation that human granulocytes had lower IL-6 receptor expression than rhesus monkey granulocytes. A similar difference was observed for granulocyte precursors in BM and also for CD34+ blast cells. It is not likely that the observed differences are caused by occupancy of IL-6 receptors by IL-6 bound in vivo because human as well as rhesus monkey cells were routinely preincubated for 1 hour at 37°C in the absence of IL-6 to promote dissociation of IL-6/receptor complexes before staining with biotin–IL-6. In addition, IL-6 receptor levels on lymphocytes and monocytes appeared to be similar between human and rhesus monkey peripheral blood. The causes of the observed differences remain to be investigated, but they could possibly be related to quantitative interspecies variations in IL-6 receptor numbers and/or binding affinity or alternatively to an increased activation status of rhesus monkey granulocytes as a result of immunologic stimulation in vivo.

The finding of significant expression of IL-6 receptors on granulocytes and monocytes would be consistent with a possible direct biologic effect of IL-6 on these cell types, as suggested by the IL-6-mediated differentiation and functional activation of normal and leukemic myeloid cells. However, the results of titration experiments indicated that freshly isolated peripheral blood monocytes and granulocytes bind IL-6 with relatively low affinity, suggesting that these cells predominantly express the biologically nonfunctional α-chain of the IL-6 receptor. The presence of the IL-6 receptor α-chain on monocytes at levels higher than on lymphocytes has previously been demonstrated in binding studies using receptor-specific antibodies and also at the mRNA level using α-chain–specific cDNA probes. Although the presence of relatively low levels of high-affinity IL-6 receptors on monocytes and granulocytes cannot be excluded, IL-6 stimulation of these cells may require additional stimuli capable of in-
Fig 4. IL-6 receptor expression on bone marrow cells. (A) Dot plot of forward-angle versus right-angle light scatter properties of Ficoll-separated rhesus monkey BM cells. The rectangular boxes indicate the electronic windows used for analysis of fluorescence data for granulocyte precursors (window G), erythroid and lymphoid cells (window E + L), and blast cells (window B). (B through D) Fluorescence profiles for the BM cell subsets identified in (A). Histograms are as indicated in the legend for Fig 1.

Expressing expression of the β subunit of the IL-6 receptor and conversion of low-affinity sites into biologically active high-affinity receptors.

The absence of detectable IL-6 receptor expression on CD19+ cells, as observed in this study, is in agreement with the absence of detectable binding of 125I-labeled IL-6 to purified B lymphocytes and the lack of direct stimulation of proliferation and differentiation of resting B lymphocytes in response to IL-6.3,20 IL-6 receptors were also not detectable on lymphocytes expressing high levels of the CD56 antigen, supporting the notion that IL-6 stimulates NK cell activities by indirect mechanisms.21 In contrast, IL-6 receptors were detected on both CD4+ and CD8+ T-lymphocytes using biotin-IL6 concentrations that were close to reported values for the dissociation constant (kd) for high-affinity IL-6 receptors (ie, ~10 to 35 pmol/L) and well below the kd values for low-affinity sites (ie, 0.7 to 2.5 nmol/L).3,2,8 These results indicated that high-affinity IL-6 receptors are present on helper T lymphocytes as well as on cytotoxic T cells. The importance of IL-6 as a costimulatory factor for antigen- or mitogen-induced stimulation of T lymphocytes has been well established.22-24 The presence of IL-6 receptors on CD8+ T lymphocytes suggests that the induction of proliferation and differentiation of cytotoxic T lymphocytes by IL-6 can be mediated via direct stimulation by IL-6, in addition to other essential signals, such as IL-2 production by helper T lymphocytes.24,23

For lack of a characterized homogeneous target cell population expressing low levels of the IL-6 receptor, it has not
been possible to determine accurately the lower limit of detection for IL-6 receptor expression using biotin-IL-6 and SA-RPE. The detection limit is also likely to be different for various cell populations because it is dependent on specific properties of cells such as their size and levels of autofluorescence. However, the results of binding studies with radiolabeled IL-6 on purified T lymphocytes indicated that T lymphocytes express, on average, approximately 200 to 300 high-affinity IL-6 receptors per cell and do not express low-affinity IL-6 receptors at all.

If an approximately fourfold difference in IL-6 receptor levels between CD4+ and CD8+ T lymphocytes (Fig 4) and an average ratio of 2.8 for CD4+ to CD8+ lymphocytes is taken into account, the numbers of IL-6 receptors can be estimated to be approximately 250 to 375/cell for the CD4+ lymphocyte subset and 60 to 90/cell for CD8+ cells. The ability to detect growth factor receptor expression on cells expressing receptor numbers as low as those present on CD8+ lymphocytes therefore demonstrates that the flow cytometric approach to detect IL-6 receptor–expressing cells is extremely sensitive.

The high-detection sensitivity and specificity of the flow cytometric method allowed for detection of IL-6 receptors on the infrequent BM cells that express CD34 and represent progenitor cells for all mature blood cell types. The frequency of CD34+ cells expressing IL-6 receptors as well as the levels of IL-6 receptor expression on these cells appeared to be lower in human as compared with rhesus monkey BM. IL-6 receptor expression on CD34+ human BM cells was examined by two-color flow cytometry using a FITC-labeled anti-CD34 MoAb, whereas IL-6 receptors on CD34+ rhesus monkey BM cells could only be detected using purified CD34+ cells because anti-CD34 MoAbs suitable for flow cytometric analysis on rhesus monkey cells were not available. The observed differences between IL-6 receptor levels on CD34+ cells in humans and rhesus monkeys could thus be caused by the different experimental approaches but also by differences in the composition of the CD34+ cell populations in both species. A more detailed comparison of the CD34+ expressing cell compartments in human and rhesus monkey BM will require the availability of anti-CD34 MoAbs that bind rhesus monkey and human CD34 with equal affinities and that can recognize cells with similar levels of CD34 expression in both species.

The results on both human and rhesus monkey CD34+ cells indicated that immature hematopoietic cells are heterogeneous with respect to IL-6 receptor expression and that some CD34+ cells do not express IL-6 receptors at detectable levels. The hemopoietic activities of IL-6 appear to include direct stimulation of the proliferation and differentiation of particularly multipotent hematopoietic cells in conjunction with other cytokines, whereas IL-6 may not be directly active on more mature lineage-committed progenitors. To further dissect the target cell specificity and hematopoietic functions of IL-6, it will thus be important to examine IL-6 receptor expression on subsets of CD34-expressing cells that differ in their differentiation stage, lineage commitment, and expression of differentiation-associated antigens, such as c-KIT, CD33, CD38, and CD71. In addition to the analysis of IL-6 receptor distribution on small subsets of immature hematopoietic cells, the flow cytometric detection of growth factor receptor expression will also be valuable to explain synergisms between IL-6 and other growth factors, in particular, IL-3 and the KIT ligand, by studying specific changes in receptor expression patterns during growth factor–stimulated proliferation and differentiation of hematopoietic cells.

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