

## REVIEW ARTICLE

# ANALYSIS OF RECEPTORS FOR CYTOKINES AND GROWTH FACTORS IN HUMAN DISEASE

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### SUMMARY

The physiological importance of cytokines and other factors which control cell and tissue growth and differentiation is widely appreciated. While physiological studies have included the cellular receptors for these factors, studies on their role in disease have concentrated on the measurement of cytokines themselves or soluble receptor components. This reflects in part the technical difficulty of measuring cell surface expression of receptors, which occur at and are functional at very low concentrations. In this review, the potential value of surface-expressed receptors as markers of disease is assessed, and methods are described which allow measurements with standard equipment for flow cytometry and fluorescence microscopy.

**KEY WORDS** Cytokine receptor Growth factor receptor Cytokine Growth factor  
Flow cytometry Microscopy

### INTRODUCTION: WHY SHOULD CYTOKINE AND GROWTH FACTOR RECEPTORS BE OF INTEREST IN HUMAN DISEASE?

Cytokines (including haematopoietic growth factors) control virtually every stage of the production, differentiation and function of the cells involved in the immune response. The factors which control the growth and differentiation of non-haematopoietic tissues, for example fibroblast growth factor, insulin-like growth factors and epithelial growth factor, play similar roles in many tissues. Indeed, there is considerable “cross-talk” between the factors of the haematopoietic tissues and the other factors, the distinction being more in the mind of the researcher than in the intrinsic properties and functions of the factors. This review will focus on the cytokines of the immune system, but the techniques and the conclusions are relevant more broadly to factors controlling tissue growth, differentiation and repair.

Recognition of the physiological and pathological roles of these molecules has been reflected in the numerous publications in which cytokine levels or cytokine mRNA are measured in a variety of normal and disease states and tissues. Since cytokines (and the other polypeptide growth factors) act through specific membrane-bound receptors, cytokine receptors must be equally important. If the receptors were constitutively expressed and functional, measurements of cytokine levels would provide an adequate

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picture of the role of a particular cytokine in a particular process. However, cytokine receptors (and other growth factor receptors) are controlled at several levels. Receptor concentrations at the membrane are modulated by signals which induce activation or differentiation, including in some cases the cytokine itself. Many of the receptors consist of two or more subunits, and the affinity of cytokine binding and the transmission of signals to the cell are dependent on assembly of the receptor complex.

Because receptors are not constitutively expressed and functional, an understanding of the role of cytokines and related factors in physiology and pathology depends as much on information about the receptors as it does on information about the ligands. Whilst the structure and physiological mode of action of the receptors is understood in some detail (Gillis, 1991; Kaczmarek and Mufti, 1991; Kishimoto *et al.*, 1992; Taga and Kishimoto, 1992; Minami *et al.*, 1993; Taniguchi and Minami, 1993), studies in disease processes have lacked balance, with the factors favoured at the expense of the receptors. Where receptors have been studied in disease, most frequently the parameter measured has been soluble receptor rather than membrane bound receptor. While circulating levels of receptor, in particular the  $\alpha$  chain of the IL-2 receptor, have provided a rather non-specific indicator of immune activation, the functional role of soluble receptors is arguable. Most soluble receptors bind cytokines with low affinity and there is little evidence that they can compete effectively with membrane-bound receptor. The best-characterised exception is the soluble IL-6 receptor, which can bind IL-6 and "deliver" it to cells expressing the signal-transducing chain CD130 (Taga *et al.*, 1989).

The major reason for the relative lack of disease association studies of cytokine receptor expression at the cell surface has been that membrane receptors are more difficult to measure than soluble receptors or the cytokines themselves. The purpose of this review is to draw attention to the likely value of information on membrane-bound receptors in understanding and monitoring disease, and to show that meaningful studies of cytokine receptors are feasible with current techniques.

The interaction between cytokines and their cellular receptors, and the subsequent reactions that lead to the effect on the cell (usually activation, differentiation or proliferation), are complex and not yet fully understood. Two components of this complexity are pleiotropy (one cytokine can act on different cells) and redundancy (one biological effect can be stimulated by any of several distinct cytokines). Cytokine pleiotropy and redundancy are explained, at least in part, by the complexity of the receptors, which consist often of multi-chain protein complexes, with some chains common to several cytokines. In many cases the cytokine binds to a protein receptor, which then associates with a second membrane protein; the latter transduces a signal to the cell (Taga and Kishimoto, 1992). The signal transduction chain can serve this function for several cytokines: gp130 for IL-6, oncostatin M, leukaemia inhibitory factor (Gearing *et al.*, 1992), ciliary neurotropic factor (Ip *et al.*, 1992; Huber *et al.*, 1993) and IL-11 (Yin *et al.*, 1993); the IL-3 receptor  $\beta$  chain for IL-3; IL-5 and GM-CSF (Lopez *et al.*, 1992); the IL-2 receptor gamma chain for IL-2 (Ishii *et al.*, 1994), IL-4 (Russel *et al.*, 1993), IL-7 (Kawahara *et al.*, 1994; Kondo *et al.*, 1994), IL-9 (Kimura *et al.*, 1995) and IL-15 (Giri *et al.*, 1994).

In this situation, abnormalities in cytokine receptors may have more profound effects than abnormalities in the cytokines themselves. An interesting and unexpected finding from the generation of "knockout" mice lacking functional genes for cytokines has been how functional a mouse lacking, for example, IL 2, can be (Rajewsky *et al.*,

1992). On the other hand, mutation in the gamma chain of the IL-2 receptor is associated with X-linked severe combined immune deficiency (Noguchi *et al.*, 1993), presumably because this mutation means that signalling by IL-2, IL-4, IL-7, IL-9 and IL-15 are all interrupted. It seems likely that abnormalities in other cytokine receptors may be associated with other immune deficiencies, and possibly, with auto immune disease and with allergy.

Cytokines can drive tumour cell proliferation, including autocrine proliferation (Sporn and Todaro, 1980; Cozzolino *et al.*, 1989), and growth factor receptors can act as oncogenes (Deuel and Huang, 1984; Cohen *et al.*, 1991; Longmore and Lodish, 1991; Wendling and Tamburin, 1991). Multiple myeloma has been characterised as a malignancy dependent on CD 130, the gp130 signal transduction molecule (Klein *et al.*, 1995), because the cytokines that act as growth factors for myeloma cells either use gp130 directly or regulate other cytokines that do. On the other hand, receptors for growth-inhibitory factors may act as tumour suppressor genes, as illustrated by the finding that mutations in the type II TGF-beta receptor are associated with colon cancer (Markowitz *et al.*, 1995).

#### WHAT IS THE PROBABLE VALUE OF QUALITATIVE AND QUANTITATIVE ANALYSIS?

Mutation of the IL-2 receptor gamma chain, in X-linked severe combined immune deficiency, or of the type II TGF-beta receptor in colon cancer, are qualitative changes — function of the receptor is absent. It is a reasonable (and testable) hypothesis that quantitative abnormalities in receptor expression may occur and may be associated with disease. If the hypothesis is correct, studies of cytokine receptor expression could provide new insights into the pathogenesis of disease and useful parameters for differential diagnosis and monitoring of disease, or monitoring of treatment. It should however be emphasised that this is a hypothesis; studies on quantitative cytokine receptor abnormalities in disease are still exploratory.

#### PHENOTYPE AND FUNCTION

Phenotypic studies are of limited value unless they are related to function. It is of little consequence that a particular cell type expresses IL-2 receptor  $\alpha$  chain if it does not respond to IL-2. Such a lack of correspondence between phenotype and function can readily be envisaged, for example, if the cell lacks one of the other chains of a multi-chain receptor, if a threshold level of stimulation is required and the receptor is expressed at too low a concentration, or if a component in the chain of intracellular signalling mechanisms triggered by engaging the cytokine receptor is missing.

On the other hand, functional studies in the absence of data on receptor expression can be misleading. IL-5 has a number of *in vitro* effects on murine B cells, but similar effects are very difficult to demonstrate with human cells, and seem not to operate in mice *in vivo*. Are the *in vitro* effects indirect? Information on receptor expression would help to understand this conundrum.

Generally, functional studies on individual patient samples as part of a diagnostic laboratory evaluation are impractical. The approach in the long term must be to evaluate the relationship between phenotype and function in a research context, and then use phenotype for individual patient studies.

## STUDIES IN NORMAL CELLS AND DISEASE ASSOCIATIONS

*Normal cells.* Many of the cytokine receptors are up-regulated by *in vitro* activation. The IL-2 receptor  $\alpha$  chain (CD25) is an extreme case, which shows rapid up-regulation in culture with a variety of stimuli. Since CD25 expression is also transient (Sayar *et al.*, 1990), CD25-positive cells in the circulation are probably cells which are in the course of, or have recently been involved in, an immune response. Consequently, circulating cells expressing some cytokine receptors may not be truly "resting" cells, although it is convenient to refer to them as unstimulated, meaning they have not been stimulated experimentally. On the other hand, some receptors are constitutively expressed, including the "common" chain, which forms part of the receptor complex for IL-2, 4, 7, 9 and 15 (Figure 1). Table 2 summarises accumulated data on the expression of several cytokine receptors by such unstimulated circulating lymphocytes. Figures 1 and 2 show two examples of studies of cytokine receptor expression on unstimulated cells.

*HIV infection.* There are at least two reasons for suggesting that CD25 monitoring in HIV infection may be useful in addition to CD4 monitoring. The first is the finding that the CD4 cells which express CD25 are the population capable of producing infective virus (Ramilo *et al.*, 1993; Borvak *et al.*, 1995). The second is that one of the first immunological functions to be depleted in HIV infection is immunological memory (Miedema, 1990). There is a positive correlation between expression of CD25 and CD45R0 (Sheldon *et al.*, 1993). Since CD45R0 is widely regarded as a marker of memory cells, while CD25 is a marker of recent activation, in that it is expressed in a transient manner (Sayar *et al.*, 1990), we have suggested that memory cells are kept in a constant state of activation by IL-2 (Sheldon *et al.*, 1993). We reported previously that CD25 expression is reduced in patients with HIV infection, and that this reduction is in part independent of CD4 reduction (Zola *et al.*, 1991).

*Other immunological disorders.* As mentioned earlier, X-linked SCID is associated with, and presumably caused by, mutation of the IL-2 receptor gamma chain, which is also a component of the receptors for IL-4, IL-7, IL-9 and IL-15. It is possible that milder forms of immune deficiency are associated with defects or quantitative deficiency of cytokine receptors, and there is at present very little information on this. Healthy newborn babies have a restricted repertoire of immune responses (Gathings and Kubagawa, 1981), and understanding the mechanism of this immunological immaturity may be helpful in understanding pathological immune deficiency. We have shown (Zola *et al.*, 1995a) that several cytokine receptors are expressed at lower levels on cord blood lymphocytes than on adult cells. The possibility that abnormalities in cytokine receptor expression may be associated with autoimmune and allergic disorders is illustrated by the elevation of IL-2 receptor  $\alpha$  chain and both components of the IL-6 receptor in systemic lupus erythematosus (Nagafuchi *et al.*, 1993).

*Cancer.* We have surveyed leukaemia cells for expression of cytokine receptors (Zola *et al.*, 1994). Figure 3 shows, as an example, the reactivity of a number of different types of leukaemia with Fas (CD95) and the receptor for IL-7. Whilst Fas is not strictly speaking a cytokine receptor, it is a structural homolog of the receptors for tumour necrosis factor (TNF) and nerve growth factor (NGF), whilst its ligand is a cell-bound homolog of TNF

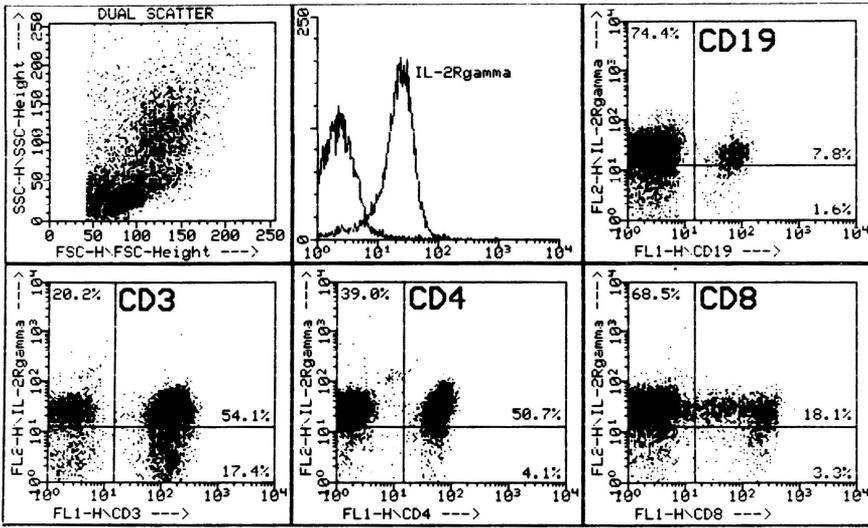


Figure 1. Expression of the IL-2 receptor  $\gamma$  chain (the common chain — common to IL-2, IL-4, IL-7, IL-9 and IL-15) by adult peripheral blood lymphoid cell populations. The top left panel shows the scatter parameters, with the lymphoid cells selected (gated—seen as a white outline). The upper centre pattern shows a fluorescence histogram for the lymphocyte population, superimposed over a negative control antibody—showing that the majority of cells express the receptor. The remaining panels show two-colour fluorescence with IL-2 receptor  $\gamma$  chain against the major lymphocyte population markers — CD19 (B cells), CD3 (T cells) and the T cell subset markers CD4 and CD8.

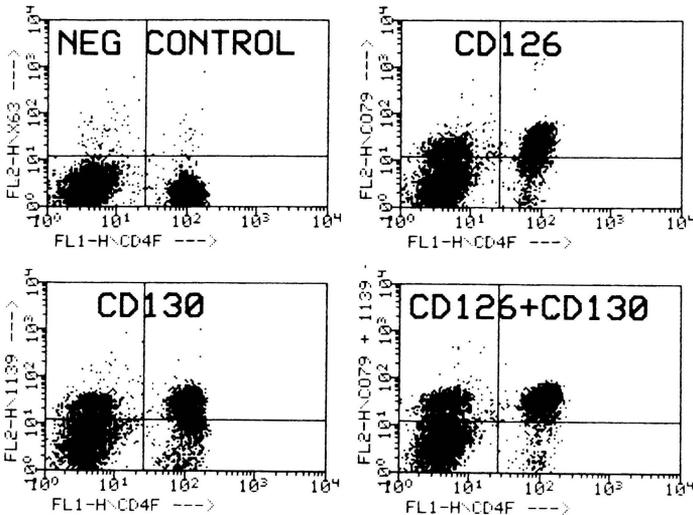


Figure 2. Expression of the IL-6 receptor (CD126) and signal transduction chain (CD130) by CD4 cells. The two-colour plots, on lymphocytes, show that the major population of cells expressing CD126 and CD130 is the CD4 population, furthermore the majority of CD4 cells express CD126 and CD130. Mixing CD126 and CD130 leads to an increase in the fraction of CD4 cells staining, indicating that a small population of cells express single receptor chains; a small number of CD4 cells (and the majority of non-CD4 cells) express neither chain. The fact that CD126 and CD130 are largely co-expressed indicates that the other cytokines that share the signal transduction chain (LIF, ciliary neurotrophic factor, oncostatin M and IL-11) are unlikely to act on significantly large additional cell populations, among blood lymphocytes.

Table 1. Monoclonal antibodies against human and murine cytokine receptors

Cytokine receptor	CD#	Supplier <sup>1</sup>
IL-1 type 1 (p80)	CD121a	Phar, Genzyme
IL-1 type 2	CD121b	Genzyme
IL-2R $\alpha$	CD25	Many
IL-2R $\beta$	CD122	BD, Phar, IT
IL-2R $\gamma$ (common chain)	*	Phar
IL-3R	CDw123	Phar
IL-3/IL-5/GMCSF common	*	Phar
IL-4R	CDw124	IT, Genzyme
IL-5R	CDw125	Phar
IL-6R	CD126	Serotec, Bio-Source, IT
gp130 (IL-6, LIF, IL11 etc.)	CD130	Serotec, Phar
IL-7R	CDw127	IT, Genzyme
TNFR p55	CD120a	Genzyme
TNFRp75	CD120b	Genzyme
IFN $\gamma$ R	CDw119	Genzyme
GMCSF	CDw116	Phar, IT
c-kit, scf-receptor	CD117	IT
FAS/APO-1	CD95	Phar, IT, COulter

# CD numbers allocated at the 5th International Workshop on Leucocyte Differentiation Antigens (Schlossman *et al.*, 1995).

\* No cluster assigned as yet.

<sup>1</sup> Commercial suppliers: BD: Becton Dickinson, San Jose, Ca, USA; Bio-S: Bio-source, Camarillo, Ca, USA; Coulter, Hialeah, Fla, USA; Genzyme, Cambridge, Ma, USA; IT: Immunotech, Marseille, France; Phar: PharMingen, San Diego, Ca, USA; Serotec, Oxford, UK.

and NGF. In general, there was a lack of correlation between receptor expression and leukaemia immunophenotype. Since immunophenotype is only a partial predictor of disease severity, cytokine receptor expression may be a useful independent prognostic marker, but a larger study, including disease outcome, is needed. We have also examined a range of cytokine receptors in solid tumours, and shown that several of the cytokine receptors do occur in solid tumours unrelated to cells of the immune system (Ridings *et al.*, 1995). Figure 4 shows a few examples. The expression of these receptors may be important in the biology of these tumours, and has implications for treatment involving cytokines.

#### MEMBRANE CONCENTRATIONS OF CYTOKINE RECEPTORS AND SENSITIVITY OF FLOW CYTOMETRY

Receptor densities are best measured by radioligand binding studies. Cells taken from blood and lymphoid tissue and not *in vitro* activated express low densities (10 to 1000 molecules per cell) of receptor. Stimulation of cells increases the level of many receptors, but densities generally range between 100 and 10,000 (Dower *et al.*, 1990). Cells expressing receptors at these levels can respond to cytokines. Thymocytes can respond

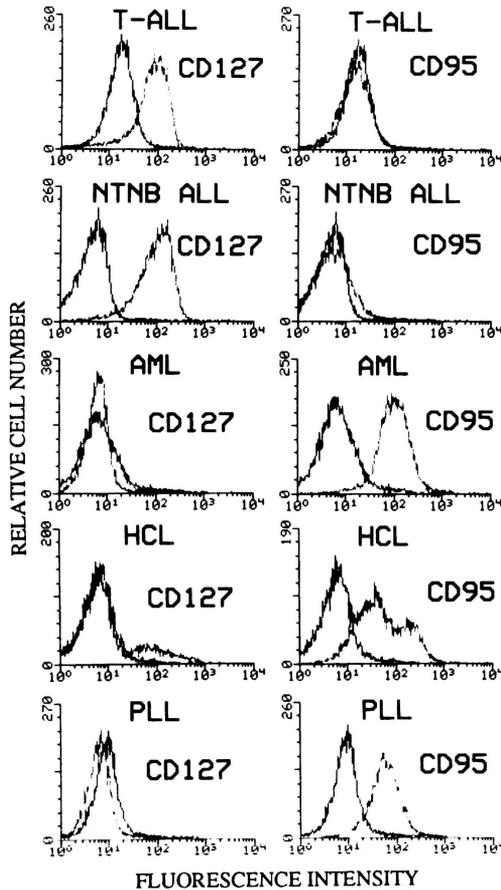


Figure 3. Expression of the IL-7 receptor (CD127) and Fas (CD95) on 5 different leukaemic cell populations. In these leukaemic samples CD127 and CD95 are expressed in a mutually exclusive pattern, with CD127 on the ALLs and CD95 on the AML and the more differentiated lymphocytic leukaemias. However, there have not been enough samples analysed to allow generalisations.

to IL-1 at concentrations of 1 to 100 molecules per cell (Dower *et al.*, 1985). IL-2 can stimulate lymphocytes without prior activation (Numerof *et al.*, 1988; Taylor *et al.*, 1986), while IL-4 can activate unstimulated B cells (Paul and Ohara, 1987).

Since cytokines can act on cells through receptors at densities between 10 and 1,000, methods are required for the analysis of receptor expression at these levels. Routine flow cytometric analysis gives clear staining of cell populations, well separated from the negative peak, with markers such as CD3, CD2, CD4, CD8 and CD19. These molecules are expressed at levels of several thousand molecules per cell. Markers present at lower levels are more difficult to detect and the limit of sensitivity of conventional flow cytometry has been put at around 2,000 (Shapiro, 1995; Zola *et al.*, 1990). CD25 is expressed at <500 copies per cell on non-activated cells (LeMauff *et al.*, 1987) and the majority of studies have detected very low numbers (3–5% or none at all) of positive cells in human peripheral blood. Studies using high-sensitivity methods have shown 30–40% of PBL cells positive (Zola *et al.*, 1989; Jackson *et al.*, 1990).

Figure 4. (Top)

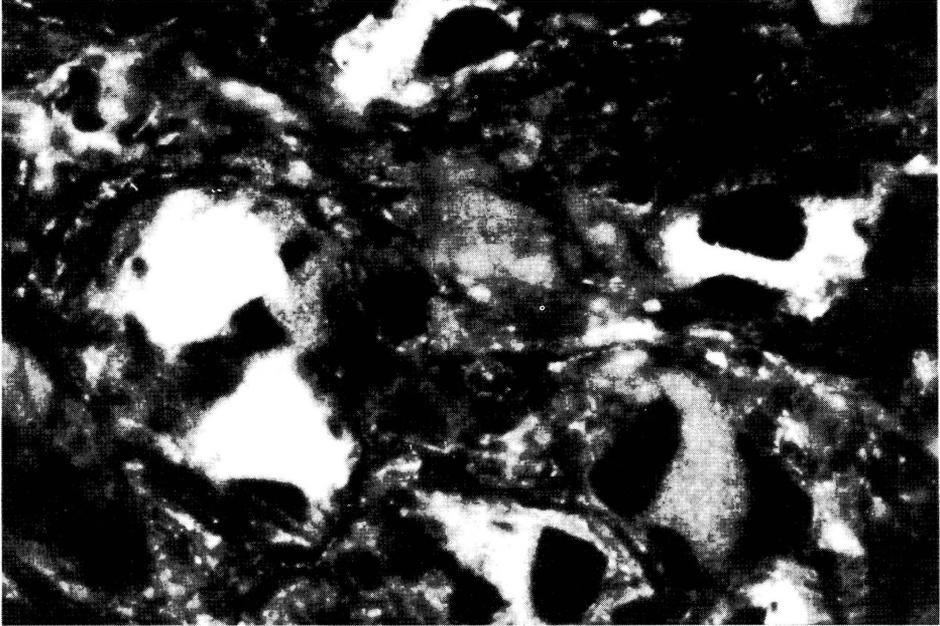


Figure 4. (Middle)

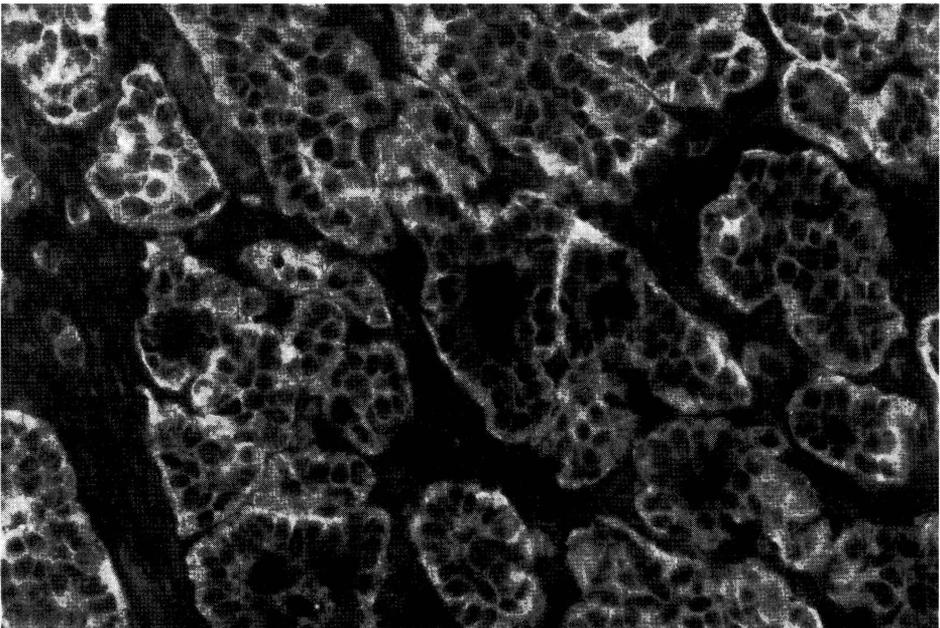


Figure 4. (Bottom)

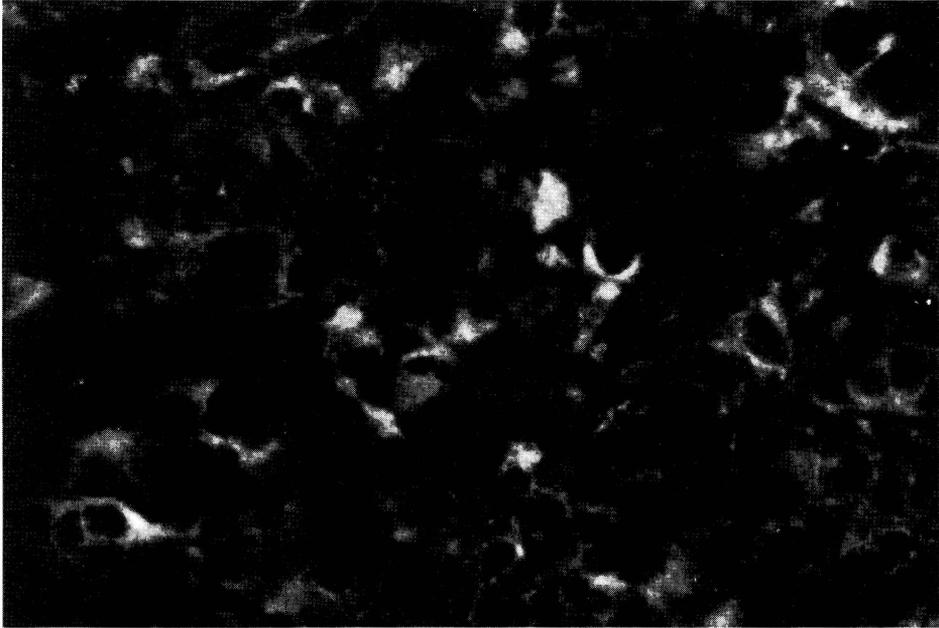


Figure 4. Expression of cytokine receptors on solid tumours. Three tumour samples stained with antibody against the gp130 signal transduction chain (CD130) shared by IL-6, LIF, ciliary neurotrophic factor, oncostatin M and IL-11. The tumours are ganlioneuroma (top panel), breast carcinoma (middle panel) and melanoma (bottom panel). A wider range of tumours and receptors has been analysed (Ridings *et al.*, 1995).

In order to achieve high staining intensities with low background staining, every aspect of the assay and sample must be considered carefully.

Fluorescein is the fluorophore most widely used but phycoerythrin (PE) is more sensitive by a factor up to 50. Each phycoerythrin molecule contains 34 fluorophore groups, giving a high extinction coefficient (the amount of light absorbed), and PE has a higher quantum yield (the efficiency of conversion of excitation energy to fluorescence energy). However, PE conjugates are variable, probably reflecting the more difficult conjugation chemistry.

Amplification is achieved by increasing the number of fluorophores per target molecule. Antibody molecules are denatured if over-conjugated and conjugates generally contain three fluorophore groups per Ig molecule. Amplification can be achieved by using extra layers — we use biotinylated anti-mouse Ig followed by PE-streptavidin. Each mouse Ig molecule will bind at least 2 anti-mouse Ig molecules; each anti-Ig molecule will have several biotin residues.

The optical parameters of the flow cytometer are less easy to manipulate, since many flow cytometers are designed to be easily operated, at the expense of flexibility. We initially obtained optimal sensitivity with a mercury arc lamp, which allowed excitation of PE at its optimal wavelength, 546 nm, rather than the standard 488nm laser line. The instrument we used for these experiments was an old model with relatively poor optics for signal collection; a combination of excitation at or near 546nm (green excitation) and

good optics should yield superior sensitivity. There have been a number of reports using green laser excitation to excite PE (Olweus *et al.*, 1993; Donahue *et al.*, 1994). We have examined the use of a green HeNe laser, a relatively inexpensive laser, but signal strength was inadequate. In a series of experiments (unpublished work of Dr. Graeme Chapman, Coulter Australia, Loretto Flego and Dr. Peter Macardle, Flinders Medical Centre, and H. Zola) we varied a number of parameters on a Coulter Epics Elite ESP cell sorter, to maximise sensitivity. The highest sensitivity, which was significantly greater than achieved on a standard flow cytometer with a fixed-wavelength 488nm laser and fixed fluidic parameters, was obtained by carefully selecting all the parameters. The use of a 76µm quartz flow cell tip and a slow flow rate increased sensitivity. The use of a 575±5nm band pass filter improved sensitivity, in spite of losing some signal, because autofluorescence was reduced, but insertion of the band-pass filter meant that photomultiplier voltage needed to be increased for maximum sensitivity. When multi-colour analysis was required, 550nm and 600nm dichroic mirrors were used to separate out the fluorescein, PE and PE/Cy5 signals. Electronic compensation for fluorescence spectral overlap reduced sensitivity significantly, so that maximum sensitivity was achieved with single colour work. When two colours were needed, the combination of PE with PE/Cy5 required less compensation than the combination of fluorescein and PE. In three-colour work, fluorescein overlapped into the PE/Cy5 channel, so that compensation was necessary. Electronic compensation and photomultiplier voltages were adjusted in an iterative procedure, since they affect each other, until maximal sensitivity, measured as the separation between negative and positive subpopulations of cells, was achieved.

Background staining has two major components: autofluorescence and non-specific binding of the reagents. Autofluorescence varies with the tissue and the wavelength measured, but it is usually a minor component of the overall background staining, as may be seen by comparing signals with and without the reagents. The intensity of autofluorescence tends to be less at the longer wavelength used to record phycoerythrin as compared with fluorescein. Non-specific staining can in turn be divided into two major components: binding of the antibodies (both the monoclonal antibody and the anti-Ig) by Fc receptors, and cross-reactivity of the anti-mouse Ig with human Ig (when the target cells are human). These different mechanisms require different solutions, and it is important to study the background staining in detail in the system under investigation (Zola, 1995). The antibody isotype and antibody "history" (whether it has been purified, frozen, chemically modified) will all affect non-specific binding. As a consequence, the inclusion of negative controls is necessary but does not guarantee that staining is specific.

Flow cytometric studies of receptors have been carried out most commonly with antibody but the labelled cytokine can be used (Armitage *et al.*, 1991). The cytokine will measure primarily high-affinity receptor, which is in several instances a compound receptor, while antibodies will detect individual receptor chains.

*Methods and reagents.* High sensitivity immunofluorescence staining for flow cytometry has been described in detail elsewhere (Zola *et al.*, 1990; Zola, 1995). The monoclonal antibody is detected by biotinylated anti-mouse Ig which is in turn detected by phycoerythrin-streptavidin (PE-SA). It is essential to use reagents which have been selected for maximum sensitivity, since biotinylated anti-mouse Ig reagents and PE-SA

vary greatly in the strength of signal produced. For 2-colour fluorescence, the unlabelled monoclonal antibody is added first, followed, after incubation and washing, by biotinylated horse anti-mouse Ig. Conjugated (fluoresceinated) antibody against CD19, CD3, CD4 or CD8 are added together with the PE-SA, after blocking available sites on the anti-mouse Ig using normal mouse Ig (Zola, 1995).

Approximately 80 monoclonal antibodies submitted as being against cytokine receptors were studied in the 5th International Leucocyte Differentiation Antigens Workshop and Conference (Schlossman *et al.*, 1995). Many of these antibodies, as well as a number of new ones, are now available through the monoclonal antibody supply companies. Table 1 lists the major specificities available.

### SENSITIVITY OF MICROSCOPY

Microscopy cannot match the quantitative precision and reproducibility of flow cytometry, but does provide invaluable data on the location of cells in relation to their environment. Furthermore, solid tissues are not always amenable to flow analysis, since disruption of the tissue may destroy cells and molecules.

Because microscopy lacks the quantitative precision of flow cytometry, it is difficult to make objective comparisons between different staining methods. Immunoenzyme staining methods are widely used, and allow good visualisation of structural detail, with the help of counterstaining. By carefully selecting reagents and using metal enhancement techniques, the sensitivity of immunoenzyme methods can be increased and can approach or equal the sensitivity of immunofluorescence (Conventry *et al.*, 1994). A comparison of immunofluorescence with metal-enhanced immunoenzymatic staining in our hands showed better detection of cells with low-level expression of IL-2 receptor by immunofluorescence (Zola *et al.*, 1995b).

As already discussed for flow cytometry, the choice of reagents, methods and equipment will greatly influence sensitivity.

*Methods and reagents.* The staining procedure follows the same principle as described above for flow cytometry, and has been described in detail recently (Zola *et al.*, 1995b). The reagents selected for flow cytometry should, in principle, be suitable for fluorescence microscopy. However, PE, the dye of choice for flow cytometry, fades very rapidly in the microscope. We have tried to get round this difficulty by using a number of anti-fading agents, by using highly sensitive light-sensitive devices and by trying to preserve fluorescence by fixing the material after staining. Whilst these methods were partially successful, fading was still too rapid to allow focussing and selection of fields for examination. The fluorochrome Cy3, on the other hand, is very slow-fading. Although it does not match the extinction coefficient or quantum yield of PE, the usable staining intensity is much better than that of PE because of the difference in fading and Cy3 is much brighter than fluorescein staining of the same material. We have tried one other dye, which shows promise. The use of immunoenzyme staining with alkaline phosphatase as the enzyme, followed by naphthol AS-MX phosphate as substrate and fast red as chromogen, gives, when illuminated with the rhodamine filter-block in a fluorescence microscope, an intense red staining, that appears not to fade. Subjectively, this staining appears to be at least as strong as that obtained with PE; however we have not been able to overcome a persistent background staining that appears to be a microcrystalline precipitate.

Table 2. Major features of expression of cytokine receptors on normal lymphocytes. For a more detailed summary, see Kikutani and Kishimoto, 1995.

Receptor	CD*	Expression	Reference
IL-1 type 1	CDw121a	No staining in normal circulating lymphocytes	Zola <i>et al.</i> , 1995c
IL-1 type 2	CDw121b	Subset of B cells	Zola <i>et al.</i> , 1995c
IL-2 $\alpha$	CD25	30–40% of circulating cells, principally CD4 cells	Zola <i>et al.</i> , 1989
IL-2 $\beta$	CD122	Most circulating cells, NK cells brightest	Zola <i>et al.</i> , 1995a
IL-2 $\gamma$	na	Most circulating cells	Ishii <i>et al.</i> , 1994
IL-3 $\alpha$	CDw123	B cell subset (weak); unidentified small population (bright)	Macardle <i>et al.</i> , 1996
IL-4	CDw124	B cells	Zola <i>et al.</i> , 1995a
GMCSF	CDw116	No staining in normal circulating lymphocytes	Zola <i>et al.</i> , 1995c
IL-3/5/GMCSF $\beta$	na	No staining in normal circulating lymphocytes	Zola <i>et al.</i> , 1995c
IL-6	CD126	Principally CD4 cells	Zola <i>et al.</i> , 1995a
gp130	CDw130	Principally CD4 cells	Zola <i>et al.</i> , 1995a
IL-7	CDw127	Principally CD4 cells	Zola <i>et al.</i> , 1995c
IL-8	CDw128	Circulating lymphocytes negative; granulocytes positive	Kikutani and Kishimoto, 1995
Stem cell factor	CD117	No staining in normal circulating lymphocytes	Zola <i>et al.</i> , 1995c
TNF type 1 p55	CD 120a	No staining in normal circulating lymphocytes	Zola <i>et al.</i> , 1995c
TNF type 2 p75	CD120b	Proportion of circulating T cells, mainly CD45R0 population	Zola <i>et al.</i> , 1995c

\*Apart from CD25, these cluster identifications were allocated at the 5th International Leucocyte Typing Workshop and Conference (Schlossman *et al.*, 1995). There were no IL-3 receptor  $\alpha$  chain antibodies in the Workshop, but the CD number CD123 was reserved for this receptor.

na: none allocated. “w” stands for “workshop”, and indicates a provisional nomenclature, allocated because only a single antibody was available for the Workshop.

In summary, our current fluorochrome of choice for microscopy is Cy3. Cy3-streptavidin is available from a number of suppliers (we use material from Jackson ImmunoResearch, West Grove, Pa.), and we use the same three-layer staining approach as described above for flow cytometry.

*Instrumentation.* The same slide can give completely different results when viewed with different optical systems. The filter blocks designed for use with rhodamine match the spectral properties of Cy3 (excitation at 552nm, maximum emission at 565nm) adequately. Objectives differ in their capacity to collect and transmit light; the numerical aperture (NA, marked on the lens barrel) is a measure of this ability. High NA lenses are usually immersion lenses, since there is a significant loss of light at glass/air interfaces. Unfortunately, low-power lenses, required to examine large field areas, are usually of low NA. It is worth experimenting with combinations of low-power eyepieces and higher power objectives to achieve maximum sensitivity. Furthermore, given the opportunity, it is worth looking at the optical efficiency of the microscope as a whole. Some of the more expensive "research microscopes" use larger optical components, for example filter blocks with a larger area, which should transmit light more efficiently. The more powerful light sources will give stronger signals. As with flow cytometry, the sensitivity depends on the difference between specific and non-specific staining, so a gain in optical efficiency may not be useful, or may require renewed attention to the staining protocol to reduce non-specific staining.

If the specimens are to be analysed by video-image analysis, the camera should be selected for sensitivity. Low-light cameras designed for security applications are suitable and relatively inexpensive; monochrome cameras are generally more sensitive than colour cameras. However, this is an area of rapidly changing technology and the suppliers should be consulted.

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