Veterinary Immunology and Immunopathology, 33 (1992) 17–24
Elsevier Science Publishers B.V., Amsterdam

Detection of canine interleukin-2 receptors by flow cytometry

Richard L. Somberg[a], J. Paul Robinson[b] and Peter J. Felsburg[a]

[a]*Department of Veterinary Pathobiology and* [b]*Department of Veterinary Physiology and Pharmacology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA*

(Accepted 8 August 1991)

ABSTRACT

Somberg, R.L., Robinson, J.P. and Felsburg, P.J., 1992. Detection of canine interleukin-2 receptors by flow cytometry. *Vet. Immunol. Immunopathol.*, 33: 17–24.

This study describes a method for detecting canine interleukin-2 receptors (IL-2R) by flow cytometry, using human recombinant IL-2 labeled with phycoerythrin (IL-2-PE). Peripheral blood mononuclear cells from four normal dogs were washed, incubated with IL-2-PE, and then washed to remove any unbound IL-2-PE. Flow cytometric analysis of the cells was performed with a 488 nm argon laser while gating on lymphocytes. Cells expressing the IL-2R were identified by their fluorescence as compared to cells stained with an anti-mouse immunoglobulin-G conjugated to phycoerythrin. The average percentage of resting cells expressing the IL-2R was found to be 21%. The addition of unlabeled human recombinant IL-2 to Day 3 phytohemagglutinin (PHA)-stimulated cells reduced the fluorescence intensity four-fold, thereby demonstrating the specificity of IL-2-PE for canine IL-2R. Following stimulation with optimal concentrations of PHA, the percentage of cells expressing the IL-2R increased daily and reached a maximum on Day 3 (76.4%). IL-2R density, as measured by mean fluorescence intensity, also increased and reached maximal levels on Days 2–3 (twenty-fold greater than resting cells). The binding, inhibition, and kinetic experiments provide evidence that human recombinant IL-2-PE is a useful tool for studying canine IL-2R expression. Thus, a one-step direct method for the flow cytometric detection and quantification of the canine IL-2R is now available.

ABBREVIATIONS

HBSS, Hank's balanced salt solution; IL-2, interleukin-2; IL-2R, interleukin-2 receptors; IL-2-PE, interleukin-2 labeled with phycoerythrin; MFI, mean fluorescence intensity; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PBS-BSA, phosphate-buffered saline–bovine serum albumin; PHA, phytohemagglutinin; XSCID, X-linked severe combined immunodeficiency.

Correspondence: Richard L. Somberg, Department of Veterinary Pathobiology, Purdue University, 1027 Lynn Hall, West Lafayette, IN 47907, USA.

INTRODUCTION

In man, interleukin-2 (IL-2) functions as a growth, differentiation and activation stimulus for cells of the T-cell lineage (Smith, 1988). The effects of IL-2 are mediated by the high-affinity or the intermediate-affinity interleukin-2 receptors (IL-2R), but not by the low-affinity IL-2R (Wang and Smith, 1987). The high-affinity IL-2R is a complex of at least two different subunits, the alpha-chain (p55 or Tac antigen/CD25) and the beta-chain (p75); the intermediate-affinity IL-2R is composed of the p75 subunit, and the low-affinity IL-2R is composed of only the p55 subunit (Waldmann, 1989). Although both subunits have IL-2 binding capacity, only the p75 subunit is able to mediate signal transduction (Robb and Greene, 1987). Monoclonal antibodies to the human and murine alpha-chain (Uchiyama et al., 1981; Malek et al., 1983) and more recently to the human beta-chain (Tsudo et al., 1989) have provided much information concerning the expression of these IL-2R subunits on resting and activated T-cells in both normal and disease states. However, the lack of monoclonal antibodies to the canine IL-2R has hampered similar studies in the dog. For example, the ability to detect canine IL-2R would be beneficial in further defining the T-cell proliferative defect in canine X-linked severe combined immunodeficiency (XSCID), a unique animal model of human XSCID (Jezyk et al., 1989).

Prior to the availability of monoclonal antibodies to the IL-2R, recombinant IL-2 labeled with ^{125}I was used to detect IL-2R (Robb et al,. 1981). Recently, IL-2 conjugated with biotin (Foxwell et al., 1988) and fluorescein-isothiocyanate (Harel-Bellan et al., 1989), have proven successful in the detection of IL-2R. Since canine T-cells proliferate in response to human IL-2 (Hogenesch and Felsburg, 1989), the canine IL-2R must be interacting with the human IL-2. In order to determine if this interaction would permit the flow cytometric detection of canine IL-2R, the binding and specificity of IL-2-PE for both resting and PHA-stimulated canine peripheral blood lymphocytes (PBL) were examined. This paper describes the results of these experiments and documents the utility of this technique for the flow cytometric detection of canine IL-2R.

MATERIALS AND METHODS

Reagents

Human recombinant IL-2 conjugated with PE at 2.5 μg ml^{-1} (IL-2-PE) was purchased from R&D Systems (Minneapolis, MN). Anti-mouse immunoglobulin G conjugated with PE (anti-IgG-PE) was purchased from Fisher Scientific (Pittsburgh, PA). Unlabeled human recombinant IL-2 at 10 000 U

ml^{-1} was purchased from Bioproducts for Science (Indianapolis, IN), Phytohemagglutinin-P (PHA), Histopaque 1.119, CPSR-2, and Histopaque 1.077 were purchased from Sigma Chemicals (St. Louis, MO).

Cells

Canine peripheral blood mononuclear cells (PBMC) were obtained from four normal dogs by a discontinuous Hypaque–Ficoll density gradient centrifugation technique developed for use in the dog (Wunderli and Felsburg, 1989). Briefly, heparinized blood was diluted 1:1 with Hank's balanced salt solution (HBSS), carefully layered onto the discontinuous density gradients in a ratio of 10 ml diluted blood: 2.0 ml of Hypaque–Ficoll 1.066: 2.0 ml of Histopaque 1.119, and centrifuged at $400\times$ *g* for 20 min at room temperature. The buoyant cells were collected and washed twice with HBSS. PHA cultures (5 $\mu g\ ml^{-1}$) were established at 1.0×10^6 cells ml^{-1} in RPMI-1640, 10% CPSR-2, antibiotics (100 U ml^{-1} penicillin G, 0.1 mg ml^{-1} streptomycin, 0.25 $\mu g\ ml^{-1}$ amphotericin B), and incubated at 37°C in a humidified 5.0% CO_2 atmosphere. Every 24 h 3.0×10^6 cultured cells were washed and centrifuged over 3.0 ml of Histopaque 1.077 to remove dead cells, and then washed twice with phosphate-buffered saline (PBS) pH 7.4, containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3 (PBS-BSA).

Flow cytometry

Washed cells (25 μl) at 4.0×10^6 cells ml^{-1} in PBS-BSA were incubated with 10 μl of IL-2-PE or 2.0 μl of anti-IgG-PE for 1 h on ice and then washed twice with PBS-BSA. The final cell pellet was resuspended in 200 μl of PBS and kept on ice until analysis on a Coulter ELITE flow cytometer (Coulter Electronics, Hialeah, FL). PE was excited using 15 mW of 488 nm argon laser light and measurement was made through a 550 long pass dichroic followed by a 625 long pass dichroic and a 575 band pass filter. As an internal control PE-conjugated beads were run each day and the mean fluorescence intensity (MFI) was set at a fixed value. Competitive inhibition of IL-2-PE staining was performed by pre-incubation of the cells with 100 μl of unlabeled human recombinant IL-2 for 30 min on ice, followed by incubation with IL-2-PE as before. Fluorescence intensity measurements were obtained by gating on the lymphocytes. Percent inhibition of MFI = 100 − (MFI + unlabeled IL-2 divided by MFI + IL-2-PE) × 100 (Taki et al., 1989).

RESULTS

Binding of human recombinant IL-2-PE to canine PBL

To examine the binding of human recombinant IL-2-PE to both resting and activated canine PBL, PBMC from four normal dogs were stimulated with optimal concentrations of PHA (5 μg ml^{-1}). The cells were stained with 10 μl of IL-2-PE prior to the addition of PHA (day 0) and every 24 h following the addition of PHA (days 1–4). Staining with 20 μl or 30 μl of IL-2-PE did not result in any significant increase in fluorescence (data not shown). The same cells were also stained with 2.0 μl of anti-IgG-PE to measure non-specific binding. As shown in Fig. 1, the fluorescence due to the binding of IL-2-PE was easily distinguished from the non-specific binding of anti-IgG-PE on both resting and PHA-stimulated PBL.

Specificity of human recombinant IL-2-PE for canine PBL

To examine the specificity of human recombinant IL-2-PE staining, a competitive inhibition assay was performed using unlabeled human recombinant IL-2. As shown in Fig. 2, pre-incubation of day 3 PBL with 100 μl of unlabeled human recombinant IL-2 significantly inhibited the binding of IL-2-PE. The

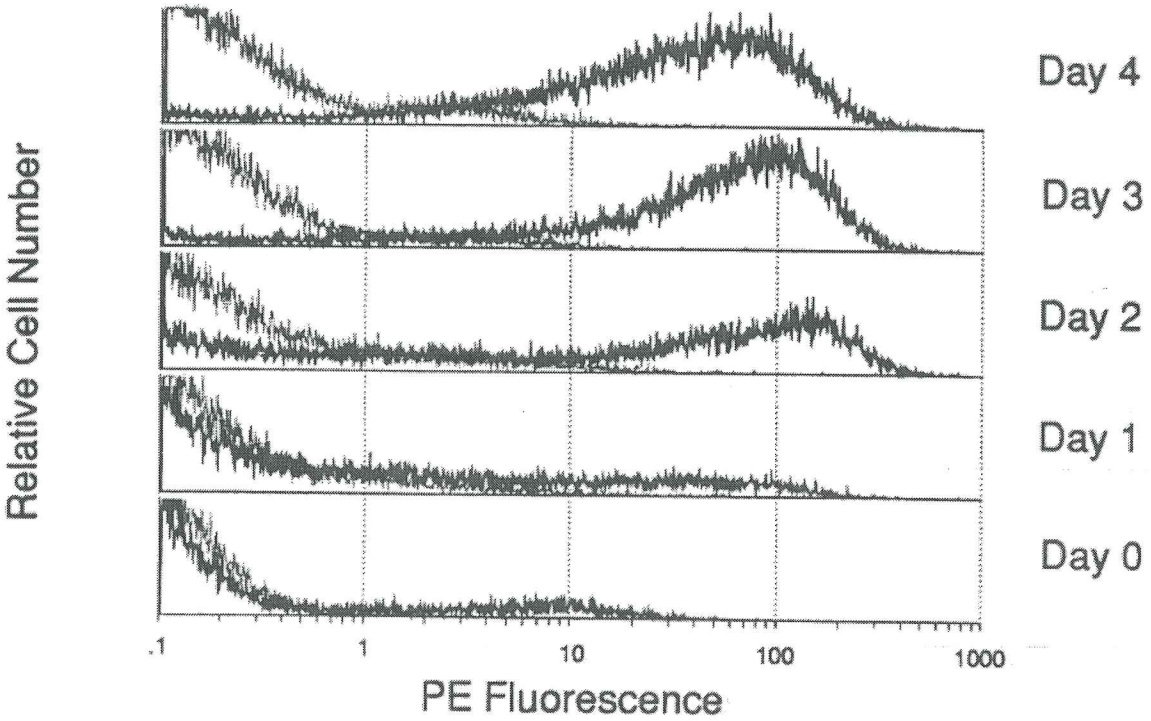

Fig. 1. Fluorescence intensity distribution of both resting and PHA-stimulated canine PBL stained with human recombinant IL-2-PE (black line) and anti-IgG-PE (gray line).

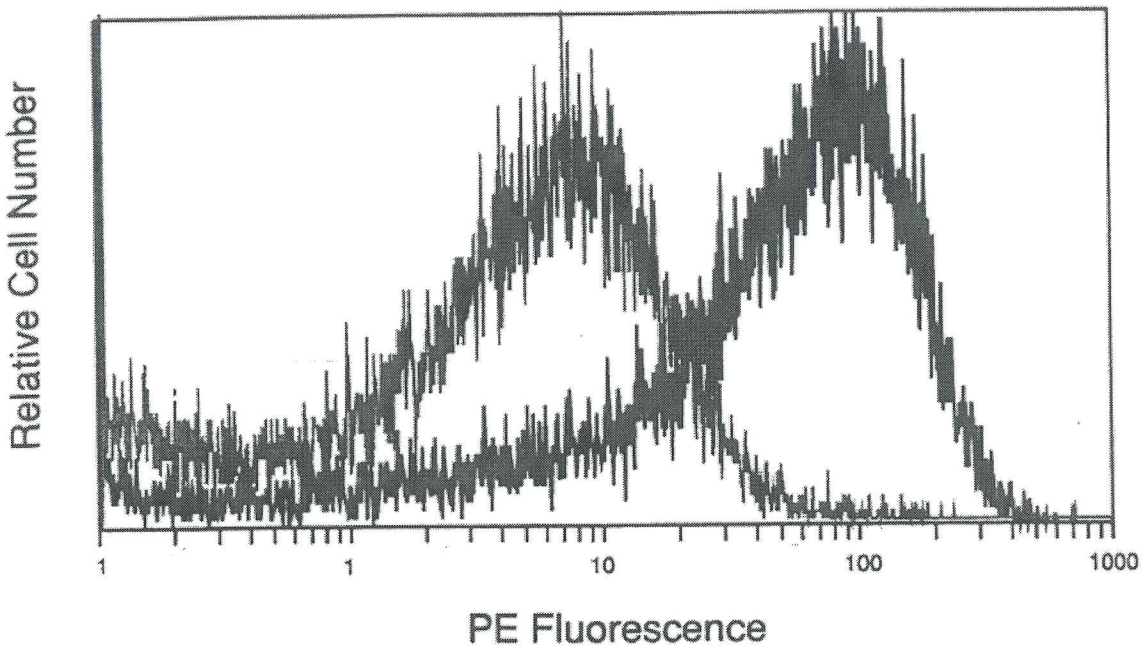

Fig. 2. Competitive inhibition of human recombinant IL-2-PE binding. Day 3 PHA-stimulated PBL were stained with IL-2-PE alone (black line) and in the presence of unlabeled human recombinant IL-2 (gray line).

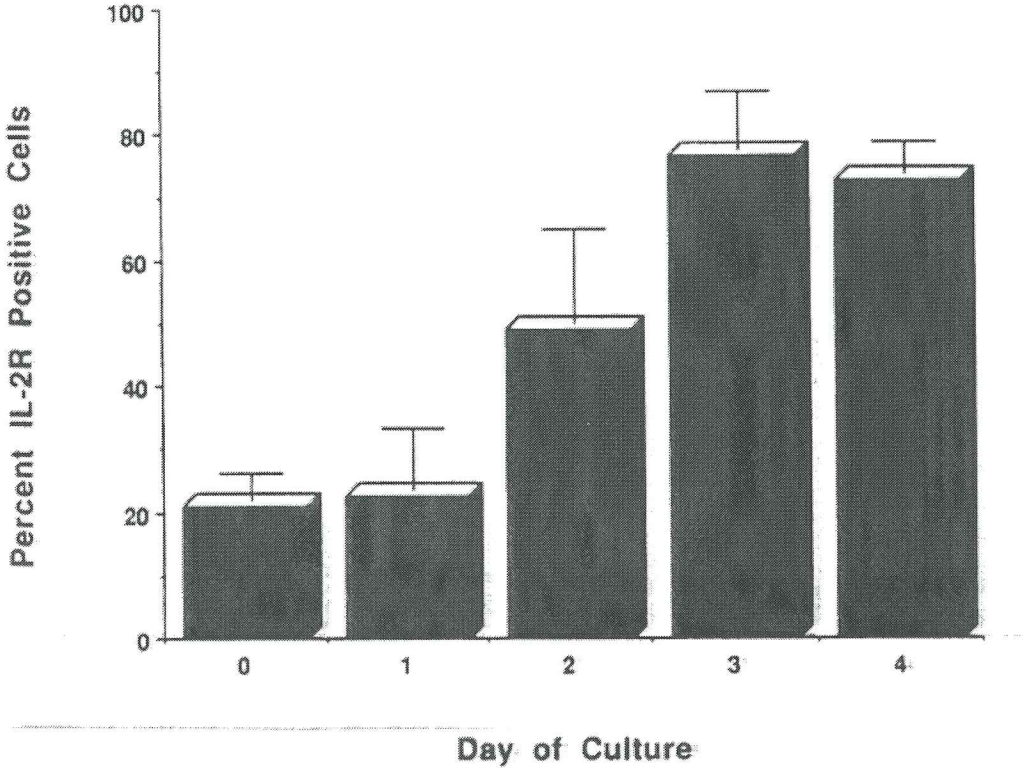

Fig. 3. Expression of IL-2R on both resting and PHA-stimulated canine PBL ($n=4$).

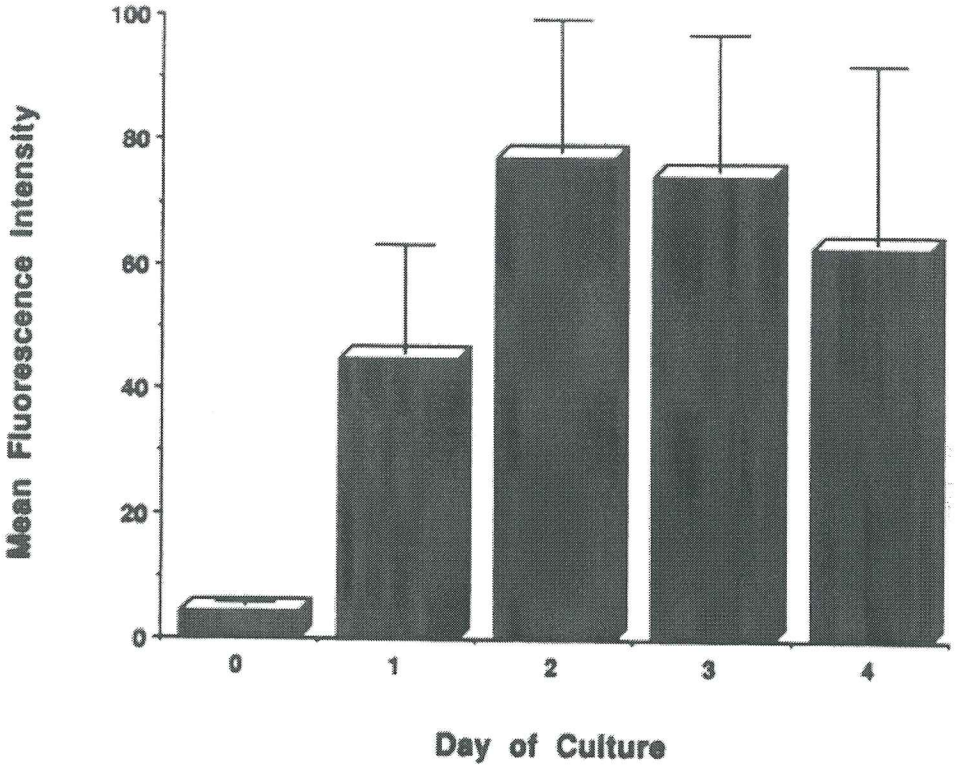

Fig. 4. Mean fluorescence intensity of IL-2R-positive cells following PHA stimulation ($n=4$).

average percent inhibition of IL-2-PE binding to PHA-stimulated canine PBL was 78.5% using the formula previously mentioned.

Kinetics of canine IL-2R expression

In order to facilitate future studies of the canine IL-2R, the kinetics of canine IL-2R expression following PHA stimulation were examined. As shown in Fig. 3, 21% of resting PBL were IL-2R positive and the percentage of IL-2R positive lymphocytes increased following PHA stimulation reaching a maximum of 76.4% by Day 3. IL-2R density, as measured by MFI, also increased following PHA stimulation and reached a maximum by Days 2–3 (Fig. 4).

DISCUSSION

Human recombinant IL-2-PE binds to both resting and PHA-stimulated canine PBL. The detection of IL-2R on 21.0% of resting canine PBL was a concern at first since few resting human PBL express the Tac antigen/CD25 (Waldmann, 1989). However, more recent studies have shown that resting human PBL do express the beta-chain (p75) of the IL-2R (Ohashi et al., 1989; Zola et al., 1990). Thus, binding of IL-2-PE to resting canine PBL may

be due to expression of a p75-like peptide. The competitive inhibition studies with unlabeled human recombinant IL-2 provide good evidence that the binding of IL-2-PE is specific for the canine IL-2R. The relatively low concentration of unlabeled IL-2 (4 ng ml^{-1}), as compared to IL-2-PE (0.2 ng ml^{-1}), perhaps was the reason that complete inhibition was not found. Studies of the kinetics of IL-2R expression following PHA stimulation revealed a dramatic increase in the percentage of IL-2R-positive cells. Furthermore, these IL-2R-positive PBL also had much higher levels of IL-2R on their cell surface as compared to resting PBL. These changes in IL-2R expression are consistent with studies performed on human PBL (Waldmann, 1989).

The inability to distinguish low- and high-affinity IL-2R and to quantify the expression of potential IL-2R subunits are disadvantages of this technique. However, Harel-Bellan et al. (1989) have shown that it is possible to distinguish low- and high-affinity IL-2R with fluorochrome-labeled IL-2 by dose-dependent binding experiments. The feasibility of this approach in the dog remains to be determined.

Until subunit-specific monoclonal antibodies or canine recombinant IL-2 become available, this technique provides the only flow cytometric method for evaluating the expression of canine IL-2R. Furthermore, this technique may be applicable to other species whose T-cells respond to human IL-2. An advantage of this technique as compared to non-flow cytometric methods is the ability to stain cells with both IL-2-PE and monoclonal antibodies for other cell surface antigens at the same time. Thus, the expression of IL-2R could be correlated with the expression, or lack of expression, of other cell surface antigens.

In summary, a one-step direct method for the detection and quantification of canine IL-2R is now available. This technique will provide a valuable tool in veterinary clinical immunology to evaluate the expression of IL-2R in immunodeficiency disease, autoimmune disease, and lymphoproliferative disease.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (AI 26103 and RR 06224) and from the American Heart Association, Indiana Affiliate.

REFERENCES

Foxwell, B.M.J., Taylor, D., Greiner, B., Mihatsch, M.J., Olivieri, V. and Ryffel, B., 1988. Biotinylated recombinant interleukin-2: a tool for research on the interleukin-2 receptor. J. Immunol. Methods, 113: 221–229.

Harel-Bellan, A., Mishal, Z., Willette-Brown, J. and Farrar, W.L., 1989. Detection of low and

high affinity binding sites with fluoresceinated human recombinant interleukin-2. J. Immunol. Methods, 119: 127–133.

Hogenesch, H. and Felsburg, P.J., 1989. Development and functional characterization of T cell lines from canine Peyer's patches. Vet. Immunol. Immunopathol., 23: 29–39.

Jezyk, P.F., Felsburg, P.J., Haskins, M.E. and Patterson, D.F., 1989. X-linked severe combined immunodeficiency in the dog. Clin. Immunol. Immunopathol. 52: 173–189.

Malek, T.R., Robb, R.J. and Shevach, E.M., 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor–ligand complex. Proc. Natl. Acad. Sci. USA, 80: 5694–5698.

Ohashi, Y., Takeshita, T., Nagata, K., Mori, S. and Sugamura, K., 1989. Differential expression of the IL-2 receptor subunits, p55 and p75 on various populations of primary peripheral blood mononuclear cells. J. Immunol., 143: 3548–3555.

Robb, R.J., Munck, A. and Smith, K.A., 1981. T-cell growth factor receptors: quantitation, specificity, and biological relevance. J. Exp. Med., 154: 1455–1474.

Robb, R.J. and Greene, W.C, 1987. Internalization of interleukin 2 is mediated by the beta chain of the high-affinity interleukin 2 receptor. J. Exp. Med., 165: 1201–1206.

Smith, K.A., 1988. Interleukin-2: inception, impact, and implications. Science, 240: 1169–1181.

Taki, S., Shimamura, T., Abe, M., Shirai, T. and Takahara, Y., 1989. Biotinylation of human interleukin-2 for flow cytometry analysis of interleukin-2 receptors. J. Immunol. Methods, 122: 33–41.

Tsudo, M., Kitamura, F. and Miyasaka, M, 1989. Characterization of the interleukin 2 receptor beta chain using three distinct monoclonal antibodies. Proc. Natl. Acad. Sci. USA, 86: 1982–1986.

Uchiyama, T., Broder, S. and Waldmann, T.A., 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. J. Immunol., 126: 1393–1397.

Waldmann, T.A., 1989. The multi-subunit interleukin-2 receptor. Annu. Rev. Biochem., 58: 875–911.

Wang, H.-M. and Smith, K.A., 1987. The interleukin-2 receptor: functional consequences of its bimolecular structure. J. Exp. Med., 166: 155–1069.

Wunderli, P.S. and Felsburg, P.J., 1989. An improved method for the isolation of enriched canine peripheral blood mononuclear cell and peripheral blood lymphocyte preparations. Vet. Immunol. Immunopathol,. 20: 335–344.

Zola, H., Purling, R.J., Koh, L.Y. and Tsudo, M., 1990. Expression of the p70 chain of the IL-2 receptor on human lymphoid cells: Analysis using a monoclonal antibody and high-sensitivity immunofluorescence. Immunol. Cell. Biol., 68: 217–224.