Daclizumab (Zenapax®) inhibits early interleukin-2 receptor signal transduction events

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Abstract

Daclizumab, a humanized antibody against the interleukin-2 (IL-2) receptor (R) α-chain, is a promising new immunosuppressant in transplantation. As its exact mechanism of action has remained unclear, we examined its short-term effects on primary human T lymphocytes expressing the high-affinity IL-2R. Daclizumab exposure for 20 min neither affected T cell viability nor their surface expression of the IL-2R α-, β-, or γ-chains. However, after IL-2 stimulation (200 U/ml, 20 min), immunoblots of cell lysates demonstrated attenuation of the IL-2-induced tyrosine phosphorylation of 65–75 kDa proteins by Daclizumab, but not by isotype controls. Since this is the molecular weight of the IL-2R β- and γ-chains, which are both tyrosine-phosphorylated by IL-2, we next examined the effect of Daclizumab on their IL-2-induced tyrosine phosphorylation. In immunoblots of IL-2R β- and γ-chain-immunoprecipitates the tyrosine phosphorylation of both chains by IL-2, but not by IL-15, was attenuated in the presence of Daclizumab. Furthermore, co-immunoprecipitation experiments showed that Daclizumab inhibited the IL-2-induced association of these chains, a prerequisite for their mutual tyrosine phosphorylation. Lastly, we demonstrated that Daclizumab inhibits the receptor-downstream induction of the IL-2-activated DNA-binding protein STAT5 in gel shift assays. We conclude that Daclizumab directly and specifically interferes with IL-2 signaling at the receptor level by inhibiting the association and subsequent phosphorylation of the IL-2R β- and γ-chains induced by ligand binding. Under our experimental conditions, Daclizumab had no effects on cell viability, and it did not modulate the surface expression of the IL-2R α-, β-, or γ-chains. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Through interaction with its receptor, interleukin-2 (IL-2) is critically involved in the clonal expansion of activated T lymphocytes, and it also mediates various important immunological processes [1]. The interleukin-2 receptor (IL-2R) is composed of three chains, and the association of all three is required for high-affinity binding of the cytokine and subsequent intracellular signaling [2]. IL-2 binding to its high-affinity receptor appears to enhance the association of the IL-2R β- and γ-chains [3,4], resulting in their mutual tyrosine phosphorylation and, subsequently, in the activation of signaling cascades which eventually induce transcription factors, such as signal transducer and activator of transcription (STAT) 5. While the β- and γ-chains are directly involved in signal transduction through their association with specific tyrosine kinases, the function of the IL-2R α-chain (CD25), which is not capable of signaling, may be to stabilize ligand binding

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and the high-affinity IL-2R [1,3]. Interestingly, CD25 is only expressed on activated T cells and is thus a possible target for immunosuppression in transplantation [5,6]. Murine antibodies against CD25 have been developed, and their efficacy in animal models lead to further modifications before their application in human transplantation. Specifically, the antigen-binding regions of these compounds were fused with human immunoglobulin (Ig) to generate 'chimeric' and 'humanized' antibodies with much longer half-lives and decreased immunogenicity [6–8]. The safety and efficacy of these compounds has now been demonstrated in clinical transplantation [9]. Of note, CD25 has so far only been identified as a component of the high-affinity IL-2R, while the IL-2R β- and γ-chains are shared by receptors for other cytokines like IL-15 [10,11]. The IL-15R, in turn, contains its own distinct α-chain [11].

Interestingly, the exact mechanism of action of humanized or chimeric anti-CD25 remains poorly understood. Based on the demonstration that the parent compound, murine anti-CD25, is non-depleting and blocks IL-2 binding to the IL-2R [6,7], it is assumed that humanized or chimeric anti-CD25s act similarly, and data exist to support this notion [12]. However, the murine IL-2R differs from the human receptor [13], and other possible actions by anti-CD25 molecules have been proposed, including some degree of depletion of CD25-positive T cells and modulation of CD25 on the cell surface [7,9]. Ravetch et al. [14] also showed that antibodies targeting molecules on the surface of T lymphocytes can not only act through binding to their specific target but also through interactions of their complement-binding fragment (Fc) with Fc receptors expressed on activated T cells. Finally, the plasma concentrations of Daclizumab required for its biological effect appear to be higher than the amount required to simply saturate CD25-positive lymphocytes [8].

2. Objectives

To clarify the mechanism of action of Daclizumab at the molecular level, we investigated early IL-2-induced signaling events in human T lymphocytes in the presence of the antibody. We examined the cells ex vivo using cell numbers and anti-CD25 concentrations resembling the clinical scenario.

3. Materials and methods

3.1. Preparation of cells

Informed consent was obtained as part of a protocol approved by the Medical Institutional Review Board of the University of Kentucky to isolate human T lympho-cytes by gradient-density centrifugation and sheep erythrocyte rosetting as described previously [15,16]. Expression of the IL-2R α-chain was induced by stimulating the T cells with 1 μg/ml of phytohemagglutinine (PHA) for 72 h at a cell concentration of 1 to 2 $\times$ 10^6/ml medium [16]. Cells were then exposed to a pH of 6.5 for 5 min, resuspended in fresh medium and rested for 24 h to allow for all PHA effects to wear off. Immediately before use, the cells were resuspended at 5×10^6/ml in RPMI containing 2% fetal calf serum. This procedure is established [17] as rendering human T cells expressing the high-affinity IL-2R and are thus most suitable for the study of IL-2R signal transduction. The final cell concentration is furthermore similar to the number of lymphocytes in the peripheral blood of young children [18]. After incubation with Daclizumab or control immunoglobulins (see below), but before application of IL-2, the cells were tested for viability by assessing their ability to exclude 0.1% Trypan Blue. All experiments were repeated with lymphocytes from different donors and with similar results.

After preparation of the cells, Daclizumab (5 μg/ml unless indicated otherwise, kindly provided by Dr. Bhupesh Desai, Hoffmann-La Roche, Inc., Nutley, NJ) was applied for 20 min. Control immunoglobulins applied instead of Daclizumab and in comparable molar concentrations consisted of human IgG1 and murine IgG2a, reflecting the immunoglobulin isotypes comprising Daclizumab [8], as well as human IgG Fc and antigen-binding (Fab) fragments (all from Chemicon, Temecula, CA). IL-2 and IL-15 (both from PeproTech, Rocky Hill, NJ) were applied at 200 U/ml and 0.5 μg/ml for 20 min, respectively, unless indicated otherwise.

3.2. Flow cytometry

Flow cytometric analysis was performed on fixed cells using fluorescein isothiocyanate- and R-phycoerythrin-conjugated isotype controls or antibodies against the human IL-2R chains essentially according to the manufacturer's instructions (Pharmingen, San Diego, CA.)

3.3. Western analysis

For immunoblots, equal amounts of cell lysates (10–20 μg per lane; BCA protein assay, Pierce Rockford, IL) were submitted to protein gel electrophoresis and transferred to a nitrocellulose membrane. After transfer, membranes were blocked and washed extensively in PBS/0.1% Tween before hybridization with appropriate antibody. Where indicated, the incubation with primary antibody against the IL-2R γ-chain was performed in the presence of saturating concentrations of a blocking peptide (Santa Cruz, CA) according to the manufacturer's instructions. For anti-phosphotyro-

sine blotting, we used PY20, a horseradish peroxidase-conjugated antibody (Transduction Laboratories, Lexington, KY). Incubation with antibodies against the IL-2R β- and γ-chains (Santa Cruz, Santa Cruz, CA) was followed by repeat washes and incubation with appropriate conjugated secondary antibody. The targeted proteins were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ), as previously described [20]. Where indicated, stained membranes were stripped in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH 6.7) for 1 h at 55°C, washed, and re-probed with the antibodies indicated in Results/Figures.

3.4. Protein immunoprecipitation

IL-2R chains were immunoprecipitated essentially as previously described by us [19,20] and others [2]. Protein-Agarose beads were coated with immunoprecipitating antibodies against the IL-2R β- and γ-chains (Santa Cruz, CA) by rocking the bead slurry with antibody in 1 ml of lysis buffer [300 mM sodium chloride, 50 mM Tris (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, 10 μg/ml of leupeptin and aprotinin, 1 mM phenylmethysulfonyl fluoride (PMSF), and 200 μM sodium vanadate] in the cold for 1 to 2 h. The beads were washed and then incubated with equal amounts of lysates overnight for immunoprecipitation. Thereafter, samples were washed five times in wash buffer (identical to lysis buffer above except for a Triton X-100 concentration of 0.1%) and boiled for 5 min in Laemmli sample buffer (BioRad, Hercules, CA) to dissociate proteins off the immunoprecipitating antibody before protein gel electrophoresis and immunoblotting.

3.5. Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were examined by incubating 20 μg of extracted protein with a radiolabeled DNA probe binding STAT5 (Santa Cruz, CA). Nuclear extracts were prepared by washing approximately 1×10^6 cells in PBS and resuspending them in 500 μl buffer A [10 mM Hepes, 10 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 mM dithiothreitol (DTT), 1 mM sodium vanadate, and 10 μg/ml leupeptin and aprotinin], followed by an incubation on ice for 10 min. Thereafter, the cells were vortexed for 10 s, spun for 10 s at 14 000 rpm, and the supernatant discarded. The pellet was resuspended in 100 μl buffer B (20 mM Hepes, 1.5 mM magnesium chloride, 0.5 mM DTT, 25% glycerol, 420 mM sodium chloride, 0.2 mM EDTA, 1 mM sodium vanadate and 10 μg/ml leupeptin and aprotinin) and incubated on ice for 20 min. Subsequent centrifugation at 14 000 rpm and 4°C for 2 min yielded a supernatant containing nuclear extract. For elec-

trophoretic mobility shift assays (EMSAs), the extracts were added to a reaction mixture containing 20 mM Tris (pH 7.5), 60 mM potassium chloride, 2 mM EDTA, 0.5 mM DTT, 2 μg poly(dI-dC), and 4% Ficoll. Unlabeled identical or unrelated double-stranded oligomers were used in 100 times molar excess for specific or non-specific competition. Protein complexes were resolved by electrophoresis on 5% non-denaturing polyacrylamide gels in $0.5 \times$ Tris-borate EDTA buffer at 12 V/cm for 2 h at room temperature. Dried gels were exposed to X-ray film (Sterling Diagnostic Imaging, Newark, DE) at −70°C.

4. Results

4.1. Cell viability

Daclizumab, applied at 5 μg/ml, did not affect cell viability as determined by Trypan Blue exclusion. In all experiments, over 95% of cells retained their ability to exclude the dye (not shown).

4.2. Dose-ranging studies

As shown in Fig. 1, the detection of Daclizumab effects in tyrosine phosphorylation assays required antibody concentrations in the higher range of therapeutic plasma concentrations [8]. Furthermore, the concentration of IL-2 required to discern any effects of Daclizumab was above 20 U/ml. Therefore, all subsequent experiments were carried out with 5 μg/ml of Daclizumab and 200 U/ml IL-2.

4.3. Effects of Daclizumab on IL-2-induced tyrosine phosphorylation events

As shown in Fig. 2, Daclizumab, but not human IgG$_1$
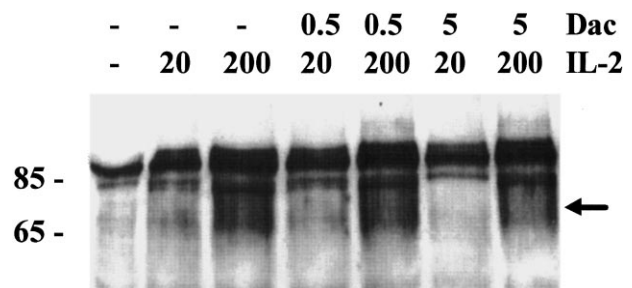

Fig. 1. Anti-phosphotyrosine immunoblot of human T cell lysates demonstrating the dose-dependent effects of Daclizumab (Dac, in μg/ml) on IL-2-induced tyrosine phosphorylation events. 5 μg/ml, but not 0.5 μg/ml, of Daclizumab attenuate protein tyrosine phosphorylation events in the 65−75 kDa M_r range induced by IL-2 given at a concentration of 200 U/ml. 20 U/ml of IL-2 produce a much weaker signal in this M_r range (arrow), and this signal is further decreased in the presence of 5 μg/ml, but not 0.5 μg/ml, of Daclizumab. M_r is given in kDa on the left.
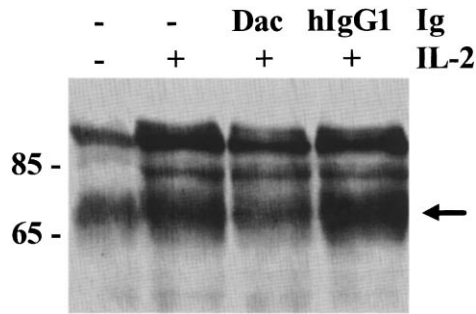
Fig. 2. Anti-phosphotyrosine immunoblot of human T cell lysates demonstrating the specific effect of Daclizumab (Dac) on IL-2-induced tyrosine phosphorylation events. Daclizumab inhibits the enhanced tyrosine phosphorylation of proteins in the 65–75 kDa M_r range by IL-2 (arrow), while human IgG1 (hIgG1) used as isotype control has no effect. M_r is given in kDa on the left.

used as Fc isotype control, attenuated IL-2-induced tyrosine phosphorylation events, especially in the 65–75 kDa M_r range. This attenuation was also undetectable when other control Ig molecules were used (not shown).

4.4. Effects of Daclizumab on IL-2-induced tyrosine phosphorylation of the IL-2R β- and γ-chains

Because the attenuation of IL-2-induced tyrosine phosphorylation events by Daclizumab was most obvious in the 65–75 kDa M_r range, we examined the effect of the antibody specifically on the tyrosine phosphorylation of the IL-2R β- and γ-chains by IL-2. The molecular weight of these chains is 70–75 and 65 kDa, respectively, and their tyrosine phophorylation by IL-2 represents a critical early event in IL-2R signal transduction (see above). Indeed, phosphotyrosine blots of both IL2R β- and γ-chain immunoprecipitates demonstrated attenuation of their tyrosine phosphorylation by IL-2 in the presence of Daclizumab (Figs. 3 and 4).
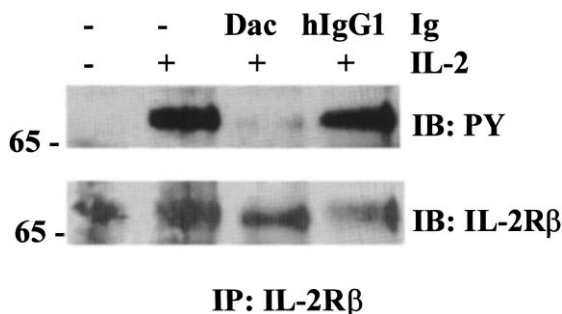

Fig. 3. Blockade of IL-2-induced tyrosine phosphorylation of the IL-2R β-chain by Daclizumab (Dac), but not by isotype control (hIgG1). IL-2R β-chain immunoprecipitates (IP) were subjected to immunoblotting (IB) with anti-phosphotyrosine antibody (PY, top panel). The membranes were subsequently stripped and re-probed with antibody against the IL-2R β-chain to confirm comparable protein loading (bottom panel). M_r is given in kDa on the left.
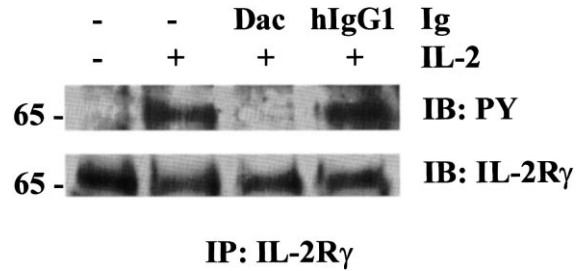

Fig. 4. Blockade of IL-2-induced tyrosine phosphorylation of the IL-2R γ-chain by Daclizumab (Dac), but not by isotype control (hIgG1). IL-2R γ-chain immunoprecipitates (IP) were subjected to immunoblotting (IB) with anti-phosphotyrosine antibody (PY, top panel). The membranes were subsequently stripped and re-probed with antibody against the IL-2R γ-chain to confirm comparable protein loading (bottom panel). M_r is given in kDa on the left.

4.5. Specificity of Daclizumab effects on IL-2-induced tyrosine phosphorylation events

To clarify whether Daclizumab specifically inhibits the IL-2-induced tyrosine phosphorylation of the β- and γ-chains shared by the IL-2R and the IL-15R, human T lymphocytes were stimulated with IL-15 instead of IL-2. As shown in Fig. 5, Daclizumab had no effect on the IL-15-induced tyrosine phosphorylation of the β- and γ-chains shared by the receptors for these two cytokines. Of note, IL-15 — but not IL-2 — also appeared to induce an altered mobility pattern of a portion of the shared receptor β-chain (cf. Figs. 3 and 5).

4.6. Effects of Daclizumab on IL-2-induced association of IL-2R β- and γ-chain

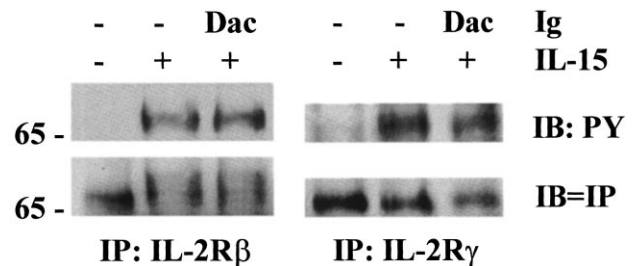It has been proposed that the tyrosine phosphorylation of the IL2R β- and γ-chains subsequent to IL-2

Fig. 5. Lack of effect of Daclizumab on IL-15-induced tyrosine phosphorylation of the β- and γ-chains shared by the IL-2- and IL-15Rs. Human T cell lysates were prepared and examined similar to those shown in Figs. 3 and 4, but after stimulation with IL-15 (0.5 μg/ml for 20 min) and not IL-2 — in the presence or absence of Daclizumab (Dac). The receptor β- and γ-chains were immunoprecipitated (IP, left panel: β-chain, right panel: γ-chain) and examined in anti-phosphotyrosine (PY) immunoblots (IB, top panels), followed by re-probing with antibody against the β- and γ-chains to confirm comparable protein loading (bottom panels). M_r is given in kDa on the left.

binding to its receptor depends on the association of the two receptor chains with each other induced by the cytokine (see above). We therefore investigated if the attenuated IL-2-induced tyrosine phosphorylation of the IL2R β- and γ-chains in the presence of Daclizumab correlated with the decreased association of the two chains. Indeed, Daclizumab decreased the amount of γ-chain that could be co-immunoprecipitated with the β-chain after IL-2 treatment (Fig. 6). Similar findings were obtained when immunoprecipitates of the γ-chain were prepared and subsequently blotted with antibody against the β-chain (not shown).

4.7. Effect of Daclizumab on the induction of STAT5 by IL-2

To document the downstream consequences of the inhibited IL-2-induced mutual association and tyrosine phosphorylation of the IL2R β- and γ-chains in the presence of Daclizumab, we performed EMSAs of T cell extracts using a radiolabeled oligonucleotide probe binding STAT5. As shown in Fig. 7, Daclizumab blocked the activation of STAT5 by IL-2.

4.8. Lack of effect of Daclizumab on expression of IL-2R α-, β- and γ-chains

To determine whether Daclizumab modulates the surface expression of any of the IL-2R chains, we determined their presence on T lymphocytes in the presence or absence of the antibody by flow cytometry. As shown in Fig. 8, Daclizumab had no effect on the amount of IL-2 R α-, β-, or γ-chain detectable on the T cell surface. Moreover, Daclizumab did not appear to interfere with the antibody used by us to detect CD25
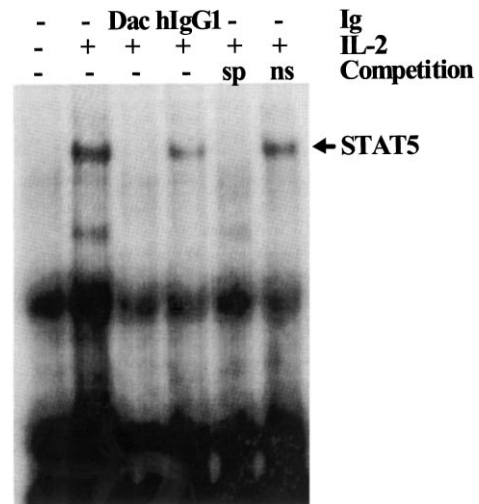

Fig. 7. EMSA demonstrating the attenuation of IL-2-induced STAT5 induction by Daclizumab (Dac), but not by isotype control (hIgG1). The specificity of the DNA-binding reaction is confirmed by competition with excess identical but unlabeled probe (specific competition, sp) or excess unrelated and unlabeled probe (non-specific, ns).

by flow cytometry, similar to its reported lack of interference with the anti-CD25 antibody 7g7 [8].

5. Discussion

We found that Daclizumab, presumably by its documented interference with IL-2 binding to its high-affinity receptor [12], attenuates the IL-2-induced association and cross-phosphorylation of the IL-2R β- and γ-chains. Accordingly, tyrosine phosphorylation events triggered by IL-2, but not by IL-15, are down-modulated, and the induction of the transcription factor STAT5 by IL-2 is dramatically reduced. These findings elucidate for the first time processes underlying the effects of anti-CD25 at the molecular level, and they document an important role of CD25 in both the formation of the high-affinity IL-2R and in the initiation of signal transduction after IL-2 binds to the receptor. Our data support and extend recent observations by Tkaczuck et al. [21], who report absent tyrosine phosphorylation of several other members of the IL-2-activated signal transduction pathways in the presence of Daclizumab, including Janus kinases (JAKs) 1 and 3, shc and also STAT5.

Our findings also emphasize the critical role of the IL-2R α-chain for the formation and function of the high-affinity IL-2R. Although the α-chain, as opposed to the β- and γ-chains, lacks an intracellular tail involved in signal transduction, it optimizes the IL-2-induced association and cross-phosphorylation of the IL-2R β- and γ-chains, presumably by allowing high affinity binding of the cytokine to the trimeric receptor. Our experiments using ex vivo primary human T lym-
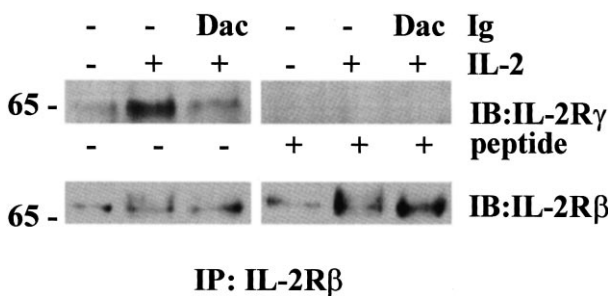

Fig. 6. Attenuation IL-2-induced association of IL-2R β- and γ-chain by Daclizumab (Dac). IL-2R β-chain immunoprecipitates (IP) were followed by immunblotting (IB) with antibody against the IL-2R γ-chain (top panel). To confirm the specificity of the antibody against the IL-2R γ-chain, two membranes were prepared with aliquots of identical immunoprecipitates and incubated with the antibody in the absence (top left panel) or presence (top right panel) of blocking IL-2R γ-chain peptide. The membranes were subsequently stripped and re-probed with antibody against the IL-2R β-chain to confirm comparable protein loading (bottom panels). M_r is given in kDa on the left.
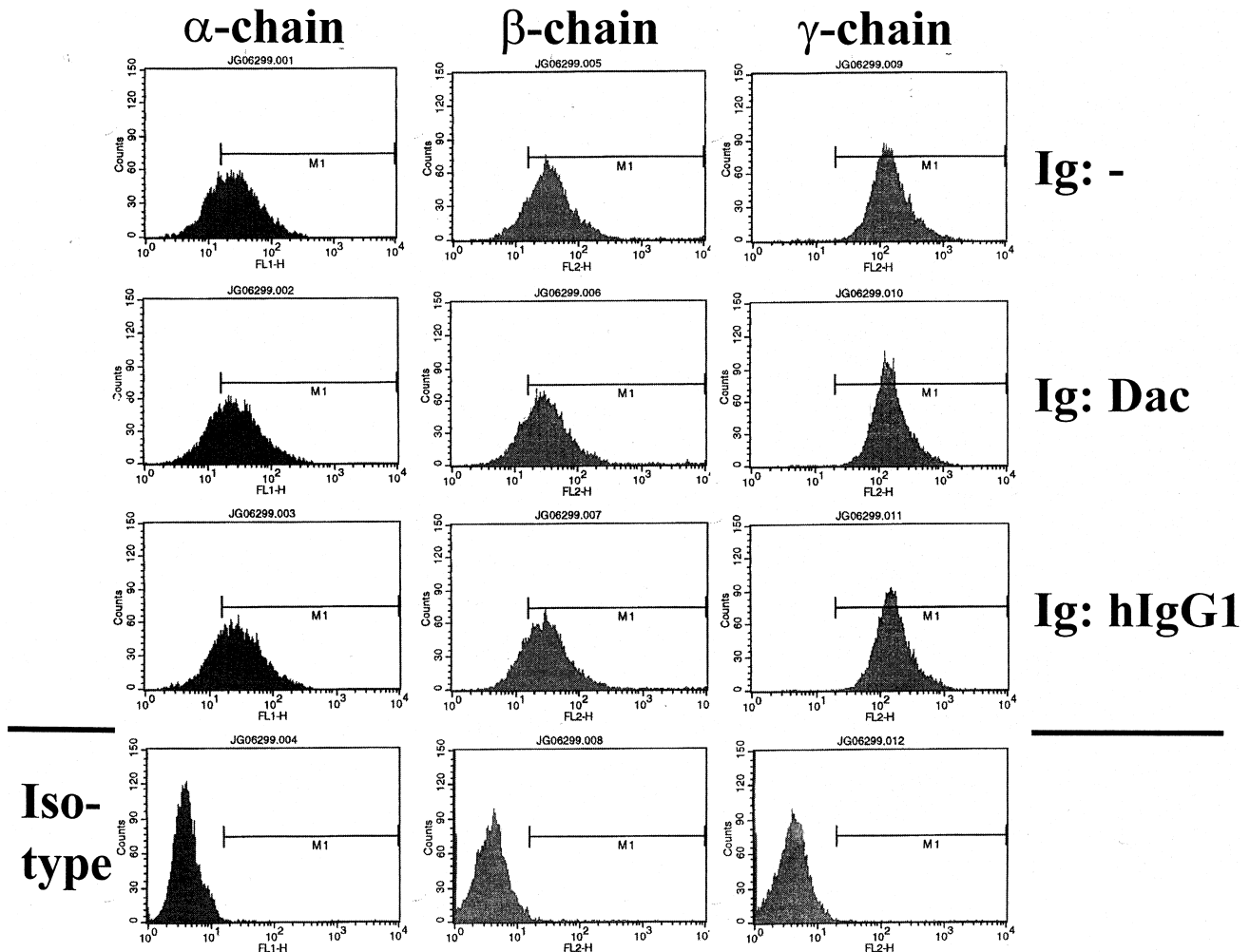
Fig. 8. Flow cytometric analysis of the expression of the IL-2R α-, β- and γ-chains on the surface of human T lymphocytes after exposure to no immunoglobulin (Ig), Daclizumab (Dac) or isotype control (hIgG1). The bottom panel represents cell staining with the appropriate labeled isotype controls for the antibodies against the α-, β- and γ-chains used for flow cytometry.

phocytes thus complement previous work by Nakamura et al. [2] and Nelson et al. [3] who reported essentially identical observations when they studied cell lines transfected with IL-2R chains. Furthermore, the effects of Daclizumab appear to be specific for the α-chain of the IL-2R, as the specific cytokine-induced tyrosine phosphorylation of the IL-15R β- and γ-chains is not affected by the antibody.

While anti-CD25 antibodies like Daclizumab are effective and safe in clinical transplantation, it needs to be emphasized that interference with IL-2 signaling is not sufficient to prevent graft rejection. As reviewed by Lakkis [22], both IL-2 and IL-2R knock-out mice are capable of mounting a graft rejection response, most likely because of the redundancy of cytokine signaling pathways. Specific evidence for such redundancy in clinical transplantation under anti-CD25 treatment has also been published [10]. Furthermore, there is now convincing evidence that IL-2, while initially providing a pro-inflammatory, i.e. 'Th1', stimulus, is eventually

important for the down-modulation of an immune response [23,24]. In fact, IL-2 and, specifically, the IL-2R α-chain appear to be required for the development of tolerance, the 'holy grail' of transplantation immunology [22,24]. Thus, these observations raise difficult questions regarding the timing and duration of anti-CD25 treatment in organ transplantation.

Lastly, we cannot completely exclude the possibility that Daclizumab and similar agents may have other effects than those described here to achieve their therapeutic potential. While we did not observe any short-term cell death induced by the reagent, it remains possible that Daclizumab may interfere with the eventual survival of CD25-positive lymphocyte subsets by impairing their IL-2-responsiveness or marking them for removal at a later time. The latter effect could be mediated by the antibody's Fc fragment, distinct from its lack of immediate effect on the initiation of IL-R signal transduction observed by us. Lastly, the absence of immediate modulations in the surface expression of

any IL-2R chains in the presence of Daclizumab does not exclude subsequent changes in their expression, especially given the comparably long half-life of the β- and γ-chains [25]. These possibilities cannot be examined in our in vitro model, and we are preparing long-term studies in transplant recipients treated with Daclizumab to address these questions.

We conclude from our experiments that at least one, if not the predominant, mechanism of action of Daclizumab is direct interference with early IL-2 signaling in human T lymphocytes. This interference occurs at the receptor level through inhibition of the association and subsequent mutual phosphorylation of the IL-2R β- and γ-chains induced by IL-2 binding. Our studies also underscore the important role of the IL-2R α-chain for the function of the high-affinity IL-2R.

Acknowledgements

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