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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Referencia : Expediente No. **11.050.626**
Asunto : Solicitud de patente para COMPOSICIÓN PARA
TRATAR ENFERMEDAD
Solicitante : BIOTEST AG
Opositor : LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.

I. ALEGATOS CONTESTACIÓN OPOSICIÓN

Yo, Luz Clemencia de Páez, en mi condición de apoderada de la sociedad solicitante BIOTEST AG, por el presente escrito doy respuesta a la oposición formulada por el doctor Tito Noé Parra, apoderado de la sociedad LABORATORIO FRANCO COLOMBIANO S.A. LAFRANCOL S.A., contra la solicitud de patente para la invención denominada COMPOSICIÓN PARA TRATAR ENFERMEDAD solicitada por mi representada en el expediente de la referencia.

II. CONTESTACIÓN DENTRO DEL TÉRMINO LEGAL

El Oficio N° 7762, que dispone el traslado de la oposición formulada fue notificado por fijación en lista el 16 de mayo de 2012, por lo que el término concedido de 60 días venció el 16 de agosto de 2012; sin embargo, teniendo en cuenta que mediante memorial radicado el

10 de agosto de 2012 mi representada solicitó una prórroga del término para presentar su defensa en este asunto, el término de la prórroga solicitada expira el 14 de noviembre de 2012, de tal manera que la oposición está siendo contestada en término legal.

III. RESPUESTA A LA PETICIÓN

Me opongo a las peticiones de la sociedad LAFRANCOL S.A., y en consecuencia solicito se declare infundada la oposición presentada en su nombre y se otorgue la patente para la invención denominada COMPOSICIÓN PARA TRATAR ENFERMEDAD solicitada por mi representada BIOTEST AG, que se tramita en este expediente.

IV. FUNDAMENTO FÁCTICO DE LA OPOSICIÓN

Fundamenta el opositor su petición, en los siguientes argumentos principales:

- 4.1. En opinión del apoderado de la sociedad opositora, la invención solicitada por mi representada en este asunto carece de los requisitos de novedad, nivel inventivo y aplicación industrial.
- 4.2. Al respecto señala que, "... las composiciones y combinaciones farmacéuticas que comprenden un agente capaz de activar las células regulatorias CD4+CD25 y Metotrexato, útiles en el tratamiento de la artritis reumatoide, son prácticas y eventos totalmente conocidos en la industria farmacéutica."

Y adicionalmente dice que, "las composiciones y combinaciones farmacéuticas que involucran moléculas como las propuestas en la presente solicitud, no presentan ninguna clase de novedad ni de nivel inventivo, luego sería inadecuado conceder un beneficio patentario."

4.3. Al respecto, llama la atención dicho apoderado acerca de la existencia de los siguientes documentos en el estado del arte:

DACLIZUMAB	The Merck Index US 5530101	1996
BASILIXIMAB	EP 449769	1991

4.4. Por los anteriores hechos se solicita negar el beneficio de patente solicitado por mi poderdante en el presente asunto.

V. RESPUESTA A LOS HECHOS Y ALEGATOS

A continuación me permito presentar a consideración de este Despacho, las razones que demuestran la improcedencia de la pretensión para que sea negado el beneficio de patente a la invención solicitada por mi representada en el expediente de la referencia.

Lo anterior, toda vez que los argumentos del apoderado de la sociedad LAFRANCOL S.A., acerca de la ausencia de los requisitos de novedad, nivel inventivo y aplicación industrial en la invención solicitada carecen de sustento fáctico y jurídico válido, ya que parten de un análisis equivocado de las normas que le sirven de sustento; por lo tanto, la oposición presentada en su nombre deberá ser declarada infundada.

5.1. En efecto, como primer aspecto se debe destacar el hecho que de acuerdo con lo previsto en el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina, **"Una invención se considerará nueva cuando no está comprendida en el estado de la técnica."**

Acerca de este requisito, el Tribunal de Justicia de la Comunidad Andina señaló en la sentencia de interpretación prejudicial del proceso 101-IP-2011, lo siguiente:

“... para que una invención sea novedosa se requiere que no esté comprendida en el estado de la técnica, es decir, que antes de la fecha de presentación de la solicitud de patente o de la prioridad reconocida, ni la innovación ni los conocimientos técnicos que de ella se desprenden hayan sido accesibles al público, entendiendo que la difusión de la información que se menciona debe ser detallada y suficiente para que una persona versada en la materia pueda utilizar esa información a fin de explotar la invención.”-se subraya-

Al respecto, la Superintendencia de Industria y Comercio ha señalado los siguientes parámetros en la guía de exámenes de solicitudes de patente para “evaluar” la novedad de una invención solicitada:

- “- Definir cuáles son las características técnicas esenciales de la primera reivindicación independiente
- Comparar, elemento por elemento, las características técnicas esenciales con las características de la materia divulgada en cada documento del estado de la técnica
- Verificar, según la comparación anterior, si la invención reivindicada es idéntica a lo revelado en el estado de la técnica, en cuyo caso se considera que no tiene novedad
- Examinar de la misma manera las otras reivindicaciones independientes, y
- Revisar si las reivindicaciones dependientes mencionan elementos nuevos.”

Tal y como podrá constatar este Despacho de acuerdo con un estudio de los argumentos de la oposición formulada, el apoderado de la sociedad LAFRANCOL S.A. se limitó a afirmar que "... las composiciones y combinaciones farmacéuticas que comprenden un agente capaz de activar las células regulatorias CD4+CD25 y Metotrexato, útiles en el tratamiento de la artritis reumatoide, son prácticas y eventos totalmente conocidos en la industria farmacéutica."

Sin embargo, dicho apoderado no demostró, a la luz de los criterios que anteriormente se destacan, que la invención de mi representada, ni los conocimientos técnicos que de ella se desprenden hayan sido accesibles al público; dicho en otras palabras, que la invención de mi representada ya hubiera sido revelada en por lo menos uno de los documentos citados como fundamento de la oposición.

A este respecto es de anotar, que la sola enumeración de una lista de documentos no es suficiente de conformidad con lo previsto en nuestra legislación vigente para desvirtuar el requisito de NOVEDAD de una invención.

Sobre el particular, la misma guía de exámenes de solicitudes de patente señala que para evaluar la novedad de una invención se "debe comparar cada reivindicación independiente con elementos del estado de la técnica y no se permite combinar elementos de más de un documento del estado de la técnica."

Sobre el particular, comedidamente solicito a este Despacho tener en cuenta lo siguiente:

En los fundamentos de la oposición se mencionan los anticuerpos Daclizumab y Basiliximab y estos se indican como descritos en las patentes US5530101 y EP0449769, respectivamente.

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Al respecto se presentan a consideración de este Despacho con este escrito, dos artículos de revistas sobre los anticuerpos identificados de la siguiente manera:

Joannes F.M. Jacobs y otros, Vacunación con células dendríticas combinada con tratamiento con anticuerpos monoclonales Anti-CD25: Un estudio de fase I/II en pacientes con melanoma metastático, Investigación clínica del cáncer, 16:5067-5078 (2010); y

T. Zhao y otros, Impacto del Basiliximab sobre la proporción de las células T reguladoras y sus subconjuntos poco después del trasplante renal: Un informe preliminar, Procedimientos de trasplantes; 44, 175-178 (2012).

Estos artículos enseñan que el Daclizumab y el Basiliximab son anticuerpos anti-CD25 (ver Resumen (Diseño experimental) de Joannes et al., y el Resumen (Objeto) de Zhao et al.).

De manera similar se advierte que la patente EP0449769 indica que la invención descrita allí está relacionada a los anticuerpos contra CD25 y que el antígeno CD25 es el nombre de la cadena α del receptor IL-2. La patente US 5530101 también hace referencia a los anticuerpos del receptor anti-IL-2 y hace referencia en particular a un anticuerpo monoclonal anti-Tac dirigido en contra del péptido Tac del receptor IL-2 humano, que también es conocido como la cadena alfa.

Las reivindicaciones de la presente solicitud están relacionadas a la combinación de un agente capaz de activar las células T reguladoras CD4+CD25+ y el metotrexato. De manera particular, la presente solicitud describe como los agentes más adecuados a los anticuerpos con uno o más dominios variables que son capaces de enlazarse a CD4 que es un objetivo diferente al descrito para Daclizumab y Basiliximab.

Adicionalmente, es de anotar que ni la patente US5530101 ni la EP0449769 parecen revelar o sugerir que los anticuerpos allí descritos son capaces de activar las células T reguladoras CD4+CD25+, según se requiere para el agente de las reivindicaciones de la presente solicitud.

De manera particular, la patente EP0449769 revela que el objetivo deseable en el tratamiento es la inactivación de las células T activadas, lo que podría estar directamente involucrado en un caso de rechazo y sugiere que este objetivo se puede alcanzar usando un anticuerpo CD25 que interfiere con el enlace de IL-2 a su receptor de alta afinidad.

Por otro lado, la patente US5530101 indica que se ha sugerido que los anticuerpos específicos del receptor IL-2, tales como el anticuerpo monoclonal anti-Tac, pueden ser usados por sí solos o como un inmunoconjugado para eliminar eficazmente las células que llevan el receptor IL-2, y que estos agentes pueden, por ejemplo, teóricamente eliminar las células T activas que expresan el receptor IL-2 involucradas en un estado de enfermedad.

Adicionalmente, a partir de la literatura de Joannes et al., y Zhao et al., parece que el Daclizumab y el Basiliximab disminuyen los números de células T reguladoras en la sangre periférica posterior a su administración, y Joannes et al. reporta que el Daclizumab agotó todas las células cd25high inmunes en su experimento.

Así, el anterior análisis permite demostrar que existe una clara y sustancial diferencia entre la invención solicitada por mi poderdante y lo revelado en los documentos citados como fundamento de la oposición.

Tal y como se señala en la guía de patentes mencionada, "Una invención no es nueva, si cada uno de sus elementos, o características, están divulgadas explícita o intrínsecamente en el estado de la

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técnica. Por tanto, hay novedad si existe cualquier diferencia entre la invención y la técnica conocida." -se subraya-

Teniendo en cuenta que en el presente caso son evidentes las diferencias entre la invención solicitada y los documentos citados por el opositor como existentes en el estado del arte, se tiene que a la luz de lo señalado en la guía de patentes y contrario a lo afirmado por este apoderado, la invención de mi representada si cumple con el requisito de novedad.

5.2. El segundo aspecto a considerar, es el requisito de NIVEL INVENTIVO que, según lo dispone el artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina, se presenta cuando **"para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."**

Sobre este requisito el Tribunal Andino de Justicia señaló en la misma sentencia de interpretación prejudicial atrás citada:

"[...] lo obvio es aquello que salta a la vista de manera clara y directa; lo "que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad". Lo evidente consiste en la "certeza clara y manifiesta de la que no se puede dudar; cierto, claro, patente y sin la menor duda."

En este orden de ideas, se puede concluir que una invención goza de nivel inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no es consecuencia clara y directa de dicho estado."

Al respecto, se ha señalado en la guía de examen de solicitudes de patentes que "La invención no tiene nivel inventivo cuando la combinación de los elementos que proveen la solución está revelada en el estado de la técnica, es decir, no basta con la existencia de los elementos de manera aislada o individualmente considerados para concluir la falta de nivel inventivo, sino que ellos en su totalidad e integridad enseñen de manera evidente la manera de llegar a la solución del problema técnico." -se subraya-

Ciertamente para la evaluación del nivel inventivo se deben señalar los documentos más cercanos del estado del arte en los cuales se revelan sugerencias y enseñanzas claras para el técnico en la materia que lo lleven a obtener la invención objeto de oposición de manera obvia y evidente, tal y como lo dispone 18 de la Decisión 486.

Sin embargo, como se dispone en la guía de exámenes de solicitudes de patente, "[...] en el examen de nivel inventivo se pueden combinar dos documentos del estado de la técnica, ó diferentes ejemplos de realización ó partes de un mismo documento, pero sólo si tal combinación fuese obvia para la persona del oficio normalmente versada en la materia técnica correspondiente.

Así, se tiene entonces que para que se pudieran considerar pertinentes las alegaciones de la parte opositora en cuanto a la ausencia de nivel inventivo de la presente invención, no basta con citar por lo menos dos documentos como los más cercanos del estado del arte, sino demostrar que sus enseñanzas revelarían al técnico de manera obvia y evidente la invención solicitada por mi poderdante en este asunto, pues es una carga que le corresponde probar y no a la administración.

Por lo tanto se tiene, que el hecho de que el apoderado de la sociedad opositora se limite simplemente a listar una serie de documentos, en que se divulgan algunas "moléculas", no es idóneo ni

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conducente para demostrar la ausencia de NIVEL INVENTIVO de la patente solicitada.

En consecuencia, se demuestra que la pretensión del apoderado a este respecto tampoco tiene vocación de prosperar, toda vez que no demostró que los documentos citados como fundamento de la oposición presentan enseñanza o sugerencia que lleve de manera obvia y evidente a obtener como resultado la invención aquí solicitada.

Por el contrario, la presente invención si tiene altura inventiva respecto a todos los documentos citados como fundamento de la oposición, pues tal y como se ha analizado en el examen de novedad de la patente solicitada en el punto 5.1 anterior, dicha invención presenta un salto cualitativo frente a lo existente en el estado de la técnica.

Al respecto me permito volver sobre las siguientes consideraciones expuestas en el examen de novedad de la presente invención acerca del hecho que Las reivindicaciones de la presente solicitud están relacionadas a la combinación de un agente capaz de activar las células T reguladoras CD4+CD25+ y el metotrexato. De manera particular, la presente solicitud describe como los agentes más adecuados a los anticuerpos con uno o más dominios variables que son capaces de enlazarse a CD4 que es un objetivo diferente al descrito para Daclizumab y Basiliximab.

Como se destacó, ni la patente US5530101 ni la EP0449769 revelan o sugieren que los anticuerpos allí descritos son capaces de activar las células T reguladoras CD4+CD25+, según se requiere para el agente de las reivindicaciones de la presente solicitud.

Por lo tanto, es evidente que la invención de mi representada al presentar un efecto sorprendente respecto a lo revelado por los documentos citados por el opositor, se tiene sin lugar a dudas que la

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invención de mi representada si cumple con el requisito de nivel inventivo.

5.3. En tercer lugar y por último, considero pertinente llamar la atención de este Despacho sobre el hecho que la presente invención si tiene aplicación industrial, contrario a lo afirmado por el apoderado de la sociedad opositora.

En efecto, una simple lectura de la descripción y la materia reivindicada en este asunto permite advertir de entrada la aplicación que tiene la invención solicitada en el sector farmacéutico, particularmente en el tratamiento de la artritis reumatode.

5.4. Por todos los anteriores hechos se tiene que, al no haber sido demostrado por el apoderado de la sociedad opositora que la invención solicitada por mi poderdante realmente carece de los requisitos de NOVEDAD, NIVEL INVENTIVO y APLICACIÓN INDUSTRIAL, conforme los presupuestos exigidos por nuestra legislación vigente, su petición no tiene vocación de prosperar, por lo que procede declarar **infundada** la oposición formulada y proseguir con el estudio de fondo de la solicitud como consecuencia de la solicitud de examen de patentabilidad oportunamente presentada en este asunto.

VI. PETICIONES

Por los anteriores hechos, solicito respetuosamente a este Despacho proceder a:

6.1. Declarar infundada la oposición presentada a nombre de la sociedad LABORATORIO FRANCO COLOMBIANO LAFRANCOL S.A.

6.2. Conceder el privilegio de patente para la invención denominada COMPOSICIÓN PARA TRATAR ENFERMEDAD a nombre de mi representada, la sociedad BIOTEST AG, que se tramita en el expediente de la referencia.

VII. PRUEBAS

Comendidamente solicito se tengan como pruebas en este asunto, todos y cada uno de los documentos que obran en el expediente de la referencia y adicionalmente las siguientes:

- 7.1. Artículo de Joannes F.M. Jacobs y otros, Vacunación con células dendríticas combinada con tratamiento con anticuerpos monoclonales Anti-CD25: Un estudio de fase I/II en pacientes con melanoma metastático, Investigación clínica del cáncer, 16:5067-5078 (2010); y
- 7.2. Artículo de T. Zhao y otros, Impacto del Basiliximab sobre la proporción de las células T reguladoras y sus subconjuntos poco después del transplante renal: Un informe preliminar, Procedimientos de transplantes; 44, 175-178 (2012).

VIII. ANEXOS

Presento con este escrito copia de los siguientes documentos:

- 8.1. Artículo de Joannes F.M. Jacobs y otros, Vacunación con células dendríticas combinada con tratamiento con anticuerpos monoclonales Anti-CD25: Un estudio de fase I/II en pacientes con melanoma metastático, Investigación clínica del cáncer, 16:5067-5078 (2010); y
- 8.2. Artículo de t. Zhao y otros, Impacto del Basiliximab sobre la proporción de las células T reguladoras y sus subconjuntos poco después del transplante renal: Un informe preliminar, Procedimientos de transplantes; 44, 175-178 (2012).

IX. NOTIFICACIONES

Mi representada y yo recibiremos notificaciones en la Secretaría de su Despacho o en mi oficina de abogado ubicada en la carrera 4ª N° 72 - 35 de esta ciudad de Bogotá, D.C.

Señor Director,


LUZ CLEMENCIA DE PÁEZ
C.C. 35.456.344
T.P.A. 23.555

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No. M-2012-229748\SCT

Clinical Cancer Research



Dendritic Cell Vaccination in Combination with Anti-CD25 Monoclonal Antibody Treatment: A Phase I/II Study in Metastatic Melanoma Patients

Joannes F.M. Jacobs, Cornelis J.A. Punt, W. Joost Lesterhuis, et al.

Clin Cancer Res 2010;16:5067-5078. Published OnlineFirst August 24, 2010.

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Dendritic Cell Vaccination in Combination with Anti-CD25 Monoclonal Antibody Treatment: A Phase I/II Study in Metastatic Melanoma Patients

Joannes F.M. Jacobs^{1,2,3}, Cornelis J.A. Punt¹, W. Joost Lesterhuis^{1,2}, Roger P.M. Suttmüller⁵, H. Mary-lène H. Brouwer², Nicole M. Scharenborg², Ina S. Klasen⁴, Luuk B. Hilbrands³, Carl G. Figdor², I. Jolanda M. de Vries^{1,2}, and Gosse J. Adema²

Abstract

Purpose: The success of cancer immunotherapy depends on the balance between effector T cells and suppressive immune regulatory mechanisms within the tumor microenvironment. In this study we investigated whether transient monoclonal antibody-mediated depletion of CD25^{high} regulatory T cells (Treg) is capable of enhancing the immunostimulatory efficacy of dendritic cell vaccines.

Experimental Design: Thirty HLA-A2.1⁺ metastatic melanoma patients were vaccinated with mature dendritic cells pulsed with tumor peptide and keyhole limpet hemocyanin (KLH). Half of the patients were pretreated with daclizumab, a humanized antibody against the interleukin-2 (IL-2) receptor α -chain (CD25), either four or eight days before dendritic cell vaccinations. Clinical and immunologic parameters were determined.

Results: Daclizumab efficiently depleted all CD25^{high} immune cells, including CD4⁺FoxP3⁺CD25^{high} cells, from the peripheral blood within four days of administration. Thirty days after administration, daclizumab was cleared from the circulation and all CD25⁺ cells reappeared. The presence of daclizumab during dendritic cell vaccinations prevented the induction of specific antibodies *in vivo* but not the presence of antigen-specific T cells. Daclizumab, however, did prevent these CD25⁺ T cells from acquiring effector functions. Consequently, significantly less patients pretreated with daclizumab developed functional, vaccine-specific effector T cells and antibodies compared with controls. Daclizumab pretreatment had no significant effect on progression-free survival compared with the control group.

Conclusions: Although daclizumab depleted the CD4⁺FoxP3⁺CD25^{high} Tregs from the peripheral circulation, it did not enhance the efficacy of the dendritic cell vaccine. Residual daclizumab functionally suppressed *de novo* induced CD25⁺ effector cells during dendritic cell vaccinations. Our results indicate that for immunotherapeutic benefit of transient Treg depletion, timing and dosing as well as Treg specificity are extremely important. *Clin Cancer Res*; 16(20): 5067–78. ©2010 AACR.

Melanoma is considered one of the most immunogenic types of cancers. This is based on the following arguments: (a) several melanoma-specific antigens have been identified (1, 2); (b) functional lymphocytes specific for melanoma antigens are increased in melanoma patients (3); (c) immune-stimulating agents can have a positive effect on

disease outcome (4, 5); and (d) spontaneous melanoma regressions with simultaneous onset of vitiligo have been reported (6).

Immunotherapeutic clinical trials have succeeded in expanding melanoma-specific effector T cells *in vivo*, but favorable outcomes are still limited because tumor-induced mechanisms of immune evasion may render the host tolerant for melanoma antigens (7, 8). Immunosuppression at the tumor microenvironment mediated by regulatory T cells (Treg) is one of the most critical mechanisms of tumor-immune escape and a major hurdle for successful immunotherapy (9–11).

In melanoma patients, selective accumulation of Tregs in the primary tumor, tumor-infiltrated lymph nodes, and in metastases has been observed (12–14). The functional importance of these Tregs is shown in mouse melanoma models in which transient Treg depletion induces antitumor immunity (15–19). These studies show that CD25 depletion effectively eliminates the CD25^{high}

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Translational Relevance

Immunity against malignant cells depends on the balance between the activity of immune effector cells and immune regulatory mechanisms. CD25⁺Foxp3⁺ regulatory T cells (Treg) prevent immune attack by directly suppressing the activation of immune effector cells. The detrimental effect of Tregs in cancer has been shown in multiple murine models in which transient depletion of Tregs enhances antitumor immunity.

In this study, we treated metastatic melanoma patients with the anti-CD25 antibody daclizumab prior to dendritic cell-based vaccination. We investigated whether the anti-CD25 antibody transiently depleted CD4⁺FoxP3⁺CD25^{high} Tregs and whether this enhanced the immunostimulatory efficacy of dendritic cell vaccines. Our results show that despite transient Treg depletion using anti-CD25 in patients, a blunted rather than enhanced T-cell response to the vaccine was observed. The data imply that for immunotherapeutic benefit, timing and dosing and especially the Treg specificity of the depleting compound are extremely important.

Tregs, although an effect on CD25⁺ effector lymphocytes has also been reported. Apparently, CD25 depletion temporarily resets the T effector/Treg balance in favor of the T effector subset. In mice several successful methodologies of Treg depletion have been described (20). This led to great interest in the depletion of Tregs as part of a multifaceted immunotherapeutic treatment of melanoma patients. However, the ability to effectively eliminate human Tregs *in vivo* has been limited by the lack of an exclusive cell surface marker. Tregs constitutively express high levels of the interleukin (IL-2) receptor (IL-2R; ref. 21). IL-2 delivers signals that are essential for the thymic development, peripheral survival, and functional fitness of Tregs (22–25). These observations have raised the question of whether drugs that interfere with IL-2 signaling might decrease the number of functional Tregs.

Denileukin diftitox (Ontak) is a recombinant IL-2/diphtheria toxin conjugate that is designed to direct the cytotoxic action of diphtheria toxin to cells that express the IL-2R (26). Thus far, clinical studies in which Ontak was administered to melanoma patients showed that it may reduce the number of circulating Tregs and cause regression of melanoma metastases (27–29).

Compared with Ontak, monoclonal antibodies (mAb) against the IL-2R α -chain (CD25) are more effective in reducing Tregs in melanoma-bearing mice (20). In addition, anti-CD25 antibodies had a synergistic effect when combined with dendritic cell-based immunotherapy in mice (20). Daclizumab (F. Hoffmann-La Roche) is a humanized antibody directed against CD25 (30).

In the present study we investigated whether treatment with a single low dose of daclizumab resulted in transient depletion of Tregs in human melanoma patients and enhancement of the immunostimulatory efficacy of subsequent dendritic cell vaccinations. We therefore vaccinated 30 HLA-A2.1⁺ metastatic melanoma patients with dendritic cells pulsed with tumor peptide and keyhole limpet hemocyanin (KLH). Half of the patients were pretreated with daclizumab.

Materials and Methods**Patient inclusion**

Inclusion criteria were metastatic melanoma with measurable disease parameters, expression of gp100 and tyrosinase in at least one metastasis, HLA-A2.1 phenotype, WHO performance status 0 or 1, no second malignancy, no serious concomitant disease, no concomitant treatment with immunosuppressive drugs, and normal hepatic and renal function. Patients with central nervous system metastases were excluded. Prior treatment related to metastatic disease was allowed, provided a treatment-free period of at least four months was observed. Patients with distant non-visceral metastases were categorized according to the American Joint Committee on Cancer (AJCC) as M_{1a}; patients with metastases to the lungs were categorized as M_{1b}, and patients with metastases to any other visceral site or with elevated serum lactate dehydrogenase were categorized as M_{1c} (31). All patients gave written informed consent prior to inclusion in the study. Approval from the local regulatory committee was obtained.

Clinical protocol and immunization schedule

In eligible patients, a leukapheresis was done from which dendritic cells were generated (32). Antigen-pulsed mature dendritic cells were administered i.v. and i.d. in close proximity to the inguinal lymph nodes three times biweekly. Patients in the daclizumab group were pretreated with 0.5 mg/kg daclizumab i.v. either 4 days (first cohort of 7 patients) or 8 days (second cohort of 8 patients) before the first dendritic cell vaccination. Patients were treated in a nonrandomized fashion. First the control group was included and treated, then the daclizumab group.

A clinical response was defined as stable disease for ≥ 4 months or any partial or complete response. Response was measured by Response Evaluation Criteria in Solid Tumors (33). Observation of progressive disease led to cessation of vaccinations. Progression-free survival (PFS) was calculated from the day of the first vaccination. Vaccine-specific immune response was the primary end point. Clinical response was the secondary end point.

Soluble IL-2R α measurement

Soluble IL-2R α (sIL-2R α) levels were determined in urine samples (U/mmol creatinine) using a commercially available immunometric assay (Immulite).

Antibodies and immunostaining

To characterize the phenotype of the *ex vivo* generated dendritic cells and the immune-cell subpopulations in the peripheral blood, flow cytometry was done using either FITC-, phycoerythrin (PE)- or allophycocyanin-conjugated mAbs. The following mAbs were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), anti-CD80 (Becton Dickinson), anti-CD14, anti-CD83 (both Beckman Coulter), anti-CD8, anti-CD25 (clone M-A251, no interference with daclizumab binding to CD25), anti-CD56, anti-CD86 (BD PharMingen), anti-Foxp3 (eBiosciences), anti-gp100 (Dako), anti-tyrosinase (Novocastra), and anti-CX3CR1 (MBL). Daclizumab antibody was covalently labeled to Alexa-488 fluor using the Alexa-488 protein labeling kit according to manufacturer's procedure (Invitrogen).

Dendritic cells: preparation, characterization, and route of administration

Dendritic cells were generated as described previously (32). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Pharmacia, 30 minutes, 4°C, 2,100 rpm) after leukapheresis. PBMCs were washed and monocytes were isolated by adherence to plastic. Monocytes were cultured at 15×10^6 per 75-cm² tissue culture flasks (Costar) in 20 mL of IL-4 (500 U/mL; Schering-Plough International), granulocyte monocyte-colony stimulating factor (800 U/mL; Schering-Plough International) and 2% human serum (HS; bloodbank Rivierenland) containing X-VIVO 15 medium (BioWhittaker). On day 6 half of the medium was replaced by autologous macrophage-conditioned medium enriched with 10 µg/mL prostaglandin E2 (Dr. G.R. Adolf, Bender & Co.) and 10 ng/mL tumor necrosis factor-α (TNF-α; kindly provided by Dr. Adolf Bender, Vienna, Austria). Cells were harvested on day 9. This procedure gave rise to mature dendritic cells as shown by high expression levels of MHC class I and II, CD80, CD83, and CD86, and the absence of CD14 (data not shown).

Cryopreservation of PBMCs and dendritic cells

All dendritic cells were cultured directly after leukapheresis and frozen as immature or mature dendritic cells for multiple vaccinations. Dendritic cells and PBMCs were frozen using a cryo 1°C freezing container (Nalgene), in freezing medium consisting of 50% X-VIVO-15 (5% HS), 40% HS albumin, and 10% DMSO. Cells were thawed in a 37°C water bath, after which the cells were washed once in cold medium and once in medium of room temperature before use.

Peptide pulsing

Dendritic cells were pulsed with wild-type peptides, gp100₁₅₄₋₁₆₇, gp100₂₈₀₋₂₈₈, and tyrosinase₃₆₉₋₃₇₆. Pulsing was done directly after harvesting or after thawing. On the day of vaccination we added peptides (50 µg/mL) for 90 minutes and kept dendritic cells at 37°C/5% CO₂. Thereafter, fresh peptides (25 µg/mL) were added, and dendritic cells were kept at room temperature for another 60 minutes. After peptide loading, dendritic cells

were washed once in 0.9% sodium chloride and resuspended in 100 µL (5×10^6 dendritic cells) for i.d. injection and in 200 µL (10×10^6 dendritic cells) for i.v. injection.

Delayed-type hypersensitivity

One week after the last of three dendritic cell vaccinations, a delayed-type hypersensitivity (DTH) skin test was done as previously described (34). Briefly, unpulsed dendritic cells, dendritic cells pulsed with gp100, dendritic cells pulsed with KLH and gp100, dendritic cells pulsed with tyrosinase, and dendritic cells pulsed with tyrosinase and KLH (10×10^5 dendritic cells each) were injected i.d. in the skin of the back of the patients at four different sites. The diameter (in millimeters) of induration was measured by a dermatologist after 48 hours, and an induration of >2 mm was considered positive. From DTH sites, punch biopsies (6 mm) were obtained under local anesthesia. Biopsies were cut in half; one part was for immunohistochemistry, whereas the other part was cut in small pieces and cultured in RPMI/7% HS supplemented with IL-2 (100 units/mL). Every 7 days, half of the medium was replaced by fresh IL-2 containing RPMI/7% HS. After 2 to 4 weeks of culturing, T cells were tested for tetramer binding, antigen recognition, and cytokine secretion.

Humoral response to KLH

Before each vaccination, 80 mL of blood were collected for immunologic monitoring. Antibodies against KLH were measured in the serum of vaccinated patients by enzyme-linked immunosorbent assay as previously described (35). Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 µg/mL) in PBS (0.1 mL/well). After washing the plates different concentrations of patient serum were added for 1 hour at room temperature. After extensive washing, specific antibodies (against IgM, total IgG, IgG1, IgG2, and IgG4) labeled with horseradish peroxidase were allowed to bind for 1 hour at room temperature. Peroxidase activity was revealed using 3,3',5,5'-tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm.

Proliferative response and cytokine production to KLH

Cellular responses against KLH were measured in a proliferation assay. Briefly, 1×10^5 PBMCs, isolated from blood samples taken before each vaccination, were plated per well of a 96-well tissue culture microplate either in the presence of KLH or without. After 16 hours of culture, supernatants (50 µL) were taken, and the levels of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ were measured by a cytometric bead array (Th1/Th2 Cytokine CBA 1, BD PharMingen) according to the manufacturer's instructions. After 4 days of culture, 1 µCi/well of tritiated thymidine was added. Incorporation of tritiated thymidine was measured in a β-counter.

MHC tetramer staining

Tetrameric-MHC complexes were kindly provided by Dr. Rosalie Luiten and Dr. Hergen Spits from the Netherlands

Cancer Institute, Amsterdam, the Netherlands. Each tetramer was validated by staining against a CTL line specific for HLA-A2 in association with the peptide of interest. PBMCs (1×10^5 cells in 10 μ L) were incubated with PE-labeled tetrameric-MHC complexes for 1 hour at room temperature. After washing, the samples were analyzed by flow cytometry.

Cytokine-secretion assay

The ability of DTH-site infiltrating lymphocytes to recognize vaccine-specific antigens and produce cytokines was measured in response to T2 cells pulsed with vaccine-specific peptides or the irrelevant HLA-A2.1 binding peptide G250. To test for recognition of endogenously processed antigens, DTH-infiltrating lymphocytes were incubated with transfected HLA-A2.1-positive BLM target cells, expressing either control antigen G250 or the antigens of study, gp100 or tyrosinase. Cytokine production was measured in supernatants after 16 hours by cytometric bead array as described above.

Autoantibody screening

Sera were screened for antinuclear antibodies by indirect immunofluorescence on HEp-2000 cells in a serum dilution of 1:40, according to the manufacturer's protocol (Biomedical Diagnostics). Sera were measured prior to start of the vaccinations and after the third vaccination.

Statistical analysis

Kaplan-Meier probability estimates of overall survival were calculated. The log rank test was used to determine statistical significance. Paired *t*-tests were done to test for treatment-related fluctuation of immune cells in the peripheral blood. Differences in KLH-specific proliferation and KLH-specific antibodies were analyzed by ANOVA. All statistical tests were two-sided, and significance was determined as $P < 0.05$.

Results

Patient characteristics

Thirty HLA-A*0201-positive patients with progressive metastatic melanoma received either daclizumab combined with dendritic cell vaccine therapy (daclizumab group; $n = 15$) or dendritic cell vaccine therapy alone (controls; $n = 15$). Patient characteristics are shown in Table 1. Patients in the daclizumab group were on average 50.9 years old (range, 25-69), which did not significantly differ from the control group (mean age, 51.7; range 22-73). The two groups were also not significantly different concerning male to female ratio, AJCC stage of the melanoma, and prior therapies.

Toxicity and side effects

No severe toxicity (common toxicity criteria grade 3-4) occurred. Vaccine-related side effects seen in patients in both groups were mild fatigue, anorexia, fever, and erythema at the intradermal injection site. None of the side

effects lasted >2 days after a vaccination. No daclizumab-related toxicity was observed. During follow-up, we did not observe any clinical signs of autoimmune disease. Because daclizumab-induced transient Treg depletion may increase the risk of inducing autoreactive antibodies, we specifically tested for the presence of antinuclear antibodies before and after vaccination. In both groups there was no significant increase of antinuclear antibodies observed (Supplementary Table S1). During follow-up, one patient in each group developed vitiligo.

Binding of daclizumab to different blood subsets

To test for the capacity of daclizumab to bind to different blood subsets, we incubated directly labeled daclizumab to erythrocyte-lysed whole blood cells of healthy controls ($n = 3$). Supplementary Fig. S1A shows that daclizumab does not bind to granulocytes (gate 1) or monocytes (gate 2) in the peripheral blood. Daclizumab binds to approximately 9% of the lymphocytes (gate 3). Daclizumab does not bind to CD20⁺ B cells, but binds to a small fraction of CD56⁺ natural killer (NK) cells, CD4⁺ Th cells, and CD8⁺ CTLs, and to a large fraction of CD4⁺FoxP3⁺ Tregs. Incubation of lymphocytes with both daclizumab-alexa⁴⁸⁸ and non-cross-reactive anti-CD25-PE showed only a double-positive and a double-negative population, which shows that daclizumab specifically and exclusively binds to CD25⁺ cells (Supplementary Fig. S1B).

Daclizumab pharmacokinetics

The pharmacokinetics of daclizumab administered to patients with metastatic melanoma is not known. Because renal clearance of sIL-2R α bound to daclizumab is blocked, measurement of sIL-2R α concentrations in the urine is an excellent predictor for daclizumab concentration in the serum (36). Analysis of urine samples showed that the sIL-2R α concentration decreased to undetectable levels immediately after daclizumab administration in all 15 patients ($P < 0.001$, paired *t*-test). Concentrations of sIL-2R α were restored in approximately 4 weeks (Fig. 1A). Daclizumab administration caused a complete and rapid depletion of all CD25^{high} cells from the peripheral blood ($P < 0.001$, paired *t*-test). Repopulation of CD25^{high} cells occurred approximately 4 weeks after daclizumab administration (Fig. 1B), as shown by using non-cross-reactive anti-CD25-PE.

Depletion of Tregs *in vivo*

Daclizumab treatment transiently depleted CD4⁺CD25^{high}FoxP3⁺ cells from the circulation (Fig. 1C and D; $P < 0.001$, paired *t*-test). The percentage of FoxP3⁺ cells was already significantly decreased 4 days after daclizumab administration (Fig. 1E; 44% reduction after 4 days, $P = 0.027$, paired *t*-test). The CD25^{high}FoxP3⁺ Tregs were especially depleted. Daclizumab did not effectively deplete the FoxP3⁺ cells with low or intermediate CD25 expression, although we cannot exclude that they were functionally impaired by bound daclizumab. Dendritic cell vaccinations without daclizumab had no effect on the

Table 1. Patient characteristics

Patient*	Age/sex	Sites of metastases	AJCC stage	Prior therapies	Prestudy LDH (U/L)	Anti-KLH response†		Tumor-specific T cells		PFS (months)
						T cell	Antibody	Blood	DTH‡	
Dac-1.	61/M	LN, skin, s.c.	M1a	S	364	+	–	–	–	3.3
Dac-2.	56/M	Lungs	M1b	S, CTx, I	357	+	–	–	–	2.5
Dac-3.	60/M	Liver	M1c	S, CTx	472	+	–	–	–	1.5
Dac-4.	28/F	LN, retroperitoneal	M1c	S	567	+	–	+§	–	2.1
Dac-5.	38/M	Lung, skin	M1c	S, CTx	451	+	–	–	–	1.8
Dac-6.	68/F	Lung, LN, skin	M1c	S	375	+	–	–	+	4.6
Dac-7.	25/M	Liver, lung, LN, skin	M1c	S	649	+	–	–	+	1.7
Dac-8.	53/F	Liver, lung, LN, skin	M1b	S, RTx	318	+	–	–	+	1.6
Dac-9.	51/M	Lung, LN, skin	M1c	S	518	+	–	–	+	1.6
Dac-10.	59/M	Liver, lung, LN	M1c	S, RTx, RF	352	+	–	–	n.t.	10.3
Dac-11.	46/M	LN	M1c	S, I	560	+	–	–	–	1.9
Dac-12.	56/M	Liver	M1c	S, RF, CTx	434	+	–	–	+	4.3
Dac-13.	69/M	Lung, skin, brain	M1c	S, RTx	735	n.t.	n.t.	n.t.	n.t.	0.7
Dac-14.	54/M	Liver	M1c	S	277	+	–	–	–	2.1
Dac-15.	40/M	Liver	M1c	S	1,289	+	–	–	n.t.	1.4
Co-1.	73/M	Lung, cut	M1b	S, RTx	355	+	+	–	–	1.5
Co-2.	56/M	Lung	M1b	S, I	302	+	+	–	n.t.	1.5
Co-3.	50/F	Intestine, s.c.	M1c	S, I	366	+	+	–	n.t.	4.5
Co-4.	66/M	LN	M1a	S	253	+	+	+	+++	13
Co-5.	35/F	Kidney, LN, spleen	M1c	S	911	+	–	–	–	4.5
Co-6.	54/M	Intestine, s.c.	M1c	S, I	389	+	+	–	–	1.5
Co-7.	22/M	Sc, testicle	M1c	S	341	–	+	–	n.t.	4
Co-8.	59/M	LN, s.c.	M1c	S, I	551	–	+	–	n.t.	2
Co-9.	33/F	LN	M1a	S	484	+	+	–	+++	114¶
Co-10.	63/M	LN, lung	M1b	S	389	+	–	–	–	2
Co-11.	72/M	Liver, LN	M1c	S	497	+	–	–	–	8
Co-12.	52/M	S.c.	M1a	S	372	n.t.	n.t.	n.t.	n.t.	0.5
Co-13.	34/M	LN, lung	M1b	S	323	+	–	–	–	2
Co-14.	56/M	Liver	M1c	S	339	+	–	–	–	6
Co-15.	51/M	Lung, s.c.	M1c	S, RTx, CTx	531	+	–	–	n.t.	2

Abbreviations: LDH, lactate dehydrogenase; CTx, chemotherapy; cut, cutaneous; I, IFN- α ; LN, lymph node; RF, radiofrequency ablation; RTx, radiotherapy; S, surgery; n.t., not tested.

*Daclizumab patients 1 to 7 received daclizumab 4 days before the first dendritic cell vaccination and patients 8 to 15 received daclizumab 8 days before the first dendritic vaccination.

†KLH-specific cellular proliferation and antibodies (total IgG) after the first vaccination.

‡Presence of tetramer-positive T cells in DTH is marked as +, presence of functional tetramer-positive T cells in DTH is marked as +++.

§Tetramer-specific T cells in the blood were also detected before vaccination.

¶Ongoing complete remission.

percentage of circulating CD4⁺FoxP3⁺CD25^{high} Tregs (Fig. 1C-E). Daclizumab administration did not significantly affect the percentage of peripheral blood CD4⁺ Th-cells, CD8⁺ CTLs, CD56⁺ NK-cells, and CD20⁺ B cells (data not shown). Daclizumab did, however, transiently increase the regulatory fraction of the NK cells (CD56^{bright}CX3XR1^{low} cells; Supplementary Fig. S2). This is consistent with previous reports showing daclizumab-

induced regulatory NK-cell expansion in patients suffering from autoimmune disease (37, 38).

Humoral responses against KLH in serum

Humoral responses against KLH were detected in serum of patients not pretreated with daclizumab. Total IgG titers were detectable in most patients already after a single vaccination with KLH-pulsed dendritic cells. After subsequent

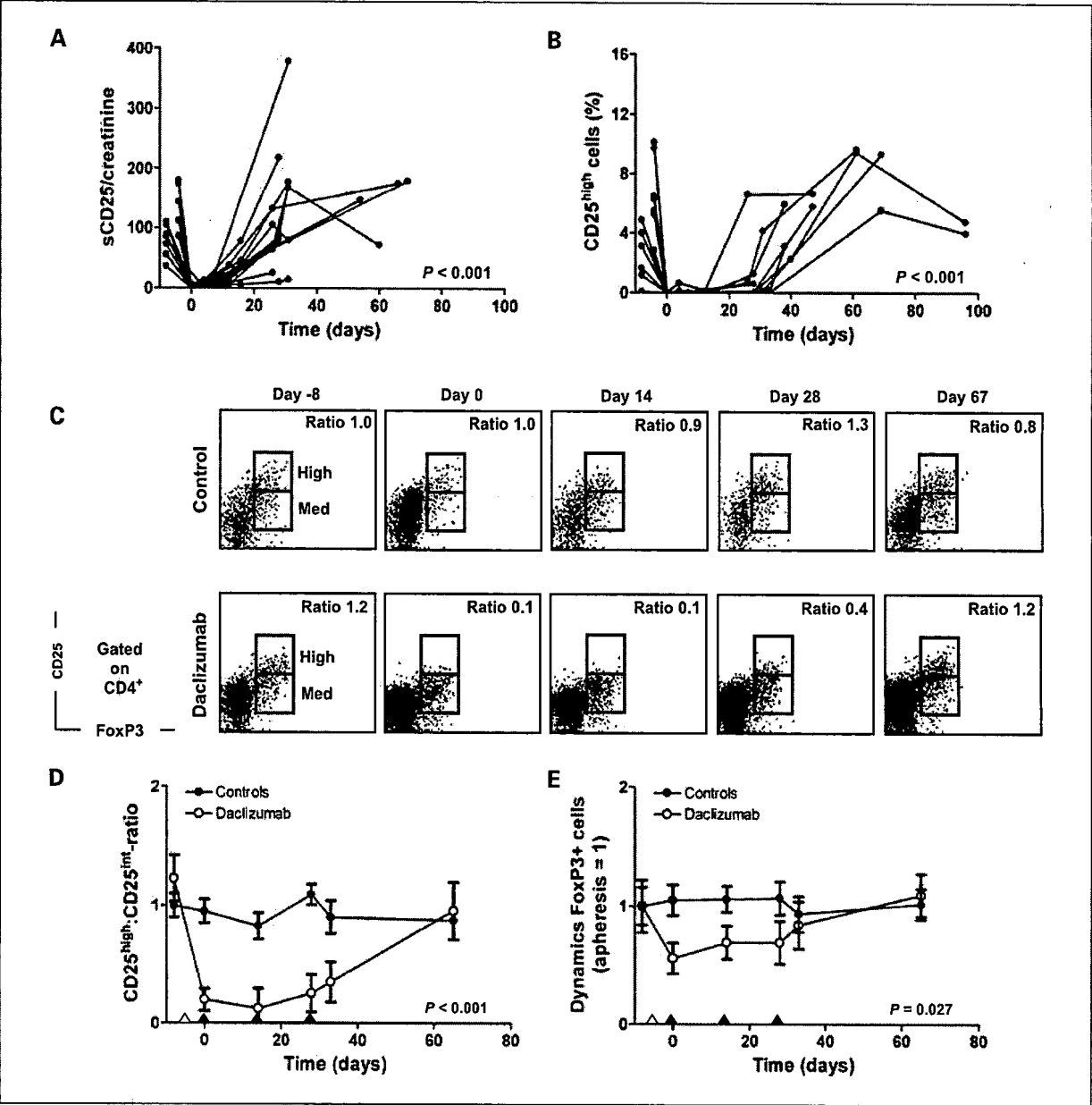


Fig. 1. Daclizumab-mediated immune effects *in vivo*. A, soluble IL-2R α levels in urine decreased to undetectable levels in all patients immediately after daclizumab administration. Restoration of soluble IL-2R α levels started three weeks after daclizumab administration. B, kinetics of CD25^{high} cells (% of total lymphocyte fraction) in the blood after daclizumab administration. C, representative dot plots on the expression of CD25 (Y-axis) and FoxP3 (X-axis) on CD4⁺-gated cells in PBMC during immunotherapeutic treatment. D, longitudinal measurement of the CD25^{high}:CD25^{intermediate} ratio of CD4⁺FoxP3⁺ cells. Δ , time of daclizumab infusion; $\blacktriangle$, dendritic cell vaccinations. Dendritic cell vaccination alone ($\bullet$; $n = 6$) does not affect the percentage of CD4⁺FoxP3⁺ CD25^{high} cells; daclizumab pretreatment ($\circ$; $n = 6$) depletes CD4⁺FoxP3⁺CD25^{high} cells. E, longitudinal measurements of the percentage of CD4⁺FoxP3⁺ cells in both control patients and patients pretreated with daclizumab.

vaccinations, the IgG antibody titers further increased (Fig. 2; ANOVA $p_{\text{after 2 vacc.}} = 0.011$; $p_{\text{after 3 vacc.}} = 0.002$). With subtype analysis of IgG antibodies we observed only IgG1 and IgG2, indicating the presence of IFN- γ and hence a Th1 response (data not shown; ref. 39). In contrast, in serum of patients pretreated with daclizumab, IgG antibodies reactive with KLH remained absent after the first dendritic

cell vaccination. After additional vaccinations, a nonsignificant increase in KLH-specific antibodies could be observed in these patients (Fig. 2). The effect of the anti-CD25 antibody, which is still present in the serum of patients during dendritic cell vaccinations (Fig. 1), on antibody production could possibly be explained by effects on the CD25⁺CD4⁺ Th cells, the CD25-expressing B cells themselves, or both.

Proliferation of PBMCs upon KLH stimulation

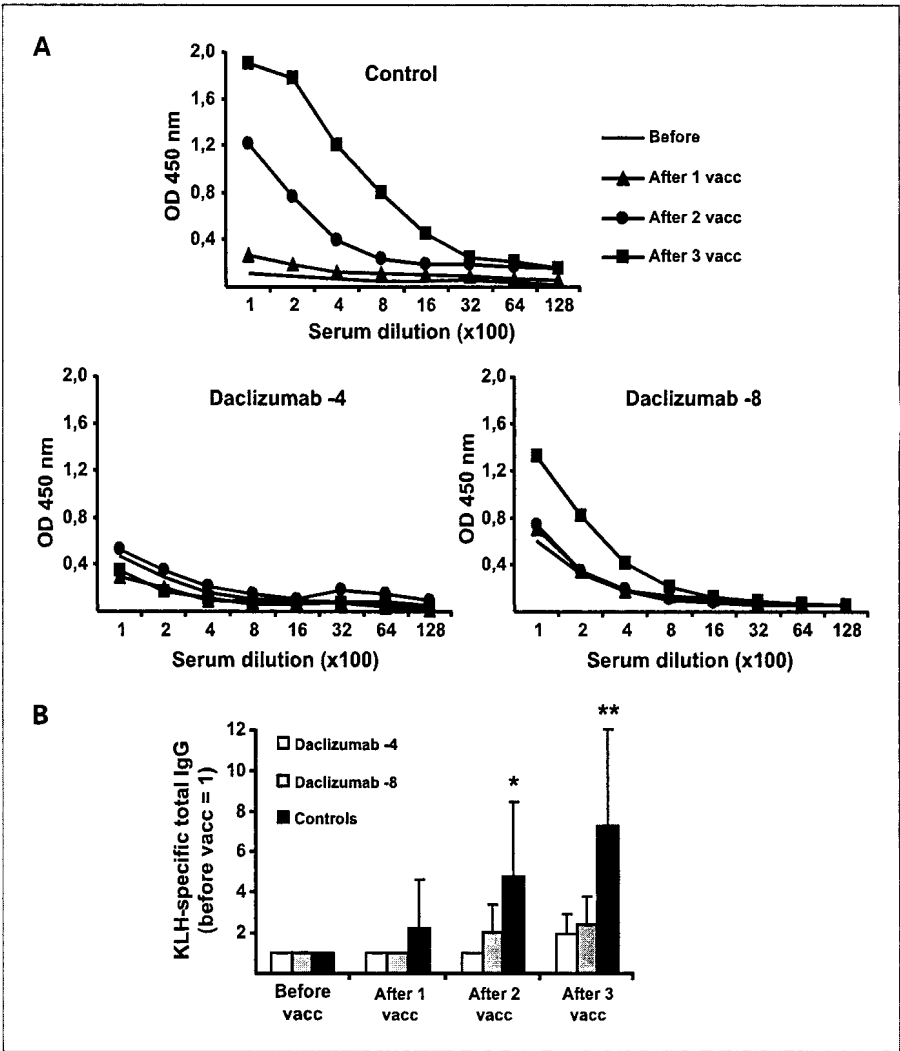
Irrespective of daclizumab treatment, all patients developed strong proliferative responses against KLH already after a single dendritic cell vaccination (Fig. 3A). During KLH-specific proliferation of PBMCs high levels of TNF- α and IFN- γ , but not IL-4, could be measured, indicating the Th1 nature of the immune response (Fig. 3B). These data show that daclizumab does not affect the generation of KLH-reactive T cells *in vivo* and these T cells are functional upon stimulation *in vitro*. To study whether the KLH-specific T-cell activation also occurs *in vivo* in the presence of daclizumab, the KLH-specific proliferative capacity of day-33 PBMCs was tested in the presence of autologous serum taken at different time points after *in vivo* daclizumab administration. Figure 3C shows that the proliferative capacity of day-33 PBMCs was almost completely abrogated when serum was added with high concentrations of daclizumab (i.e., a few days after daclizumab administration). Even after 5 weeks the daclizumab con-

centration in the serum of this patient was high enough to reduce proliferation with 40% compared with serum without daclizumab (i.e., day -4). No inhibition of KLH-specific proliferation was observed with serum from control patients (data not shown). These data suggest that in patients pretreated with daclizumab, vaccine-induced KLH-specific CD4⁺ Th cells are generated but are functionally impaired as long as daclizumab is present.

Detection of tumor-specific T cells *in vivo*

To determine the effect of daclizumab on the presence of tumor-associated antigen-specific CD8⁺ T cells, tetramer stainings were done after three vaccinations (Fig. 4A and Supplementary Fig. S3A). In peripheral blood, tetramer-positive cells could be seen in only two patients (Dac-4 and Co-4). In patient Dac-4 the tetramer-positive T cells were also detectable in the blood prior to dendritic cell vaccinations. In both groups, significantly more antigen-specific CD8⁺ T cells were found in short-term T-cell

Fig. 2. Antibodies against KLH *in vivo*. A, representative total IgG antibodies against KLH in the serum of a control patient [dendritic cell vaccination (vacc) alone], a patient pretreated with daclizumab at day -4, and a patient pretreated with daclizumab at day -8. B, results of total IgG against KLH of all patients vaccinated with either dendritic cells alone (black bars), dendritic cells with daclizumab pretreatment day -4 (white bars), and dendritic cells with daclizumab pretreatment day -8 (grey bars). A significant increase in total IgG titers was detected only in control patients (ANOVA $p_{\text{after 2 vacc.}} = 0.011$; $p_{\text{after 3 vacc.}} = 0.002$).



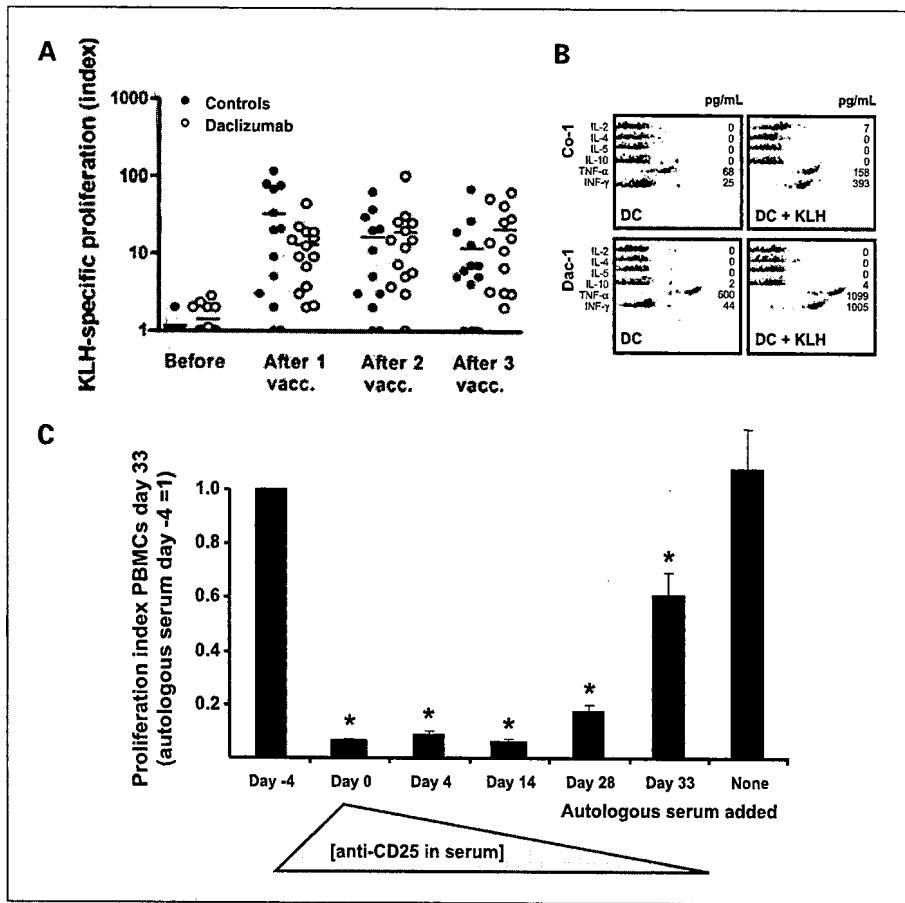


Fig. 3. KLH-specific proliferation of PBMCs before and after vaccination. **A**, both controls (●) and daclizumab patient-derived PBMCs (○) show a strong proliferative response against KLH that could already be detected after a single dendritic cell vaccination. **B**, cytokine production by PBMCs of representative patients after three dendritic cell (DC) vaccinations. **C**, KLH-specific proliferation of PBMCs after three dendritic cell vaccinations (day 33) in the presence of autologous serum obtained at various time points (patient Dac-1).

cultures (2-4 weeks) from biopsies of positive DTH reactions. In 2 of 10 tested control patients antigen-specific T cells were detected. The antigen-specific CD8⁺ T cells in these two patients were fully functional and produced high levels of IL-2 and IFN- γ upon antigen-specific stimulation (Supplementary Fig. S3B). In patients pretreated with daclizumab, we detected antigen-specific T cells in 5 of 13 evaluable patients. Strikingly, in 4 of 5 patients these tetramer-positive CD8⁺ T cells were completely non-functional and did not produce any cytokines upon antigen-specific stimulation. The gp100₁₅₄-tetramer-specific cells of patient Dac-8 produced low amounts of IL-2, IL-5, and IFN- γ upon stimulation with the gp100₁₅₄ peptide but not upon stimulation with the endogenously expressed gp100 protein (Fig. 4B and C). In conclusion, vaccine-induced CD8⁺ T cells in the daclizumab group were present after the dendritic cell vaccinations. In contrast to the CD4⁺ Th cells, however, these CD8⁺ T cells were functionally impaired *in vitro* even in the absence of daclizumab.

Clinical outcome

No significant difference was seen in PFS between patients that received daclizumab at day -4 or day -8 (Table 1). Analyzed as one group, patients that received

daclizumab prior to dendritic cell vaccinations had the same PFS compared with patients that received only dendritic cell vaccinations (Fig. 5 and Table 1). Both the immunomonitoring data and the clinical follow-up indicate that daclizumab administration prior to dendritic cell vaccination does not enhance the efficacy of the vaccine. Survival in both the daclizumab group and the control group was comparable with what has been reported in the literature for a population of patients with metastatic melanoma (40). Although the number of patients in this study with specific T cells was limited and did not allow statistical analysis, the data are in accordance with our previous work (34) showing that those patients with functional antigen-specific T cells after vaccination have a prolonged PFS (Table 1). Despite the presence of specific T cells in five patients within the daclizumab group, their inability to respond to tumor cells most likely explains the lack of impact on PFS in these patients.

Discussion

Immune-suppressive CD4⁺FoxP3⁺CD25^{high} Tregs accumulate in primary and metastatic melanomas, which undermines spontaneous and/or vaccine-induced immune responses against melanomas (12-14). The aim of this

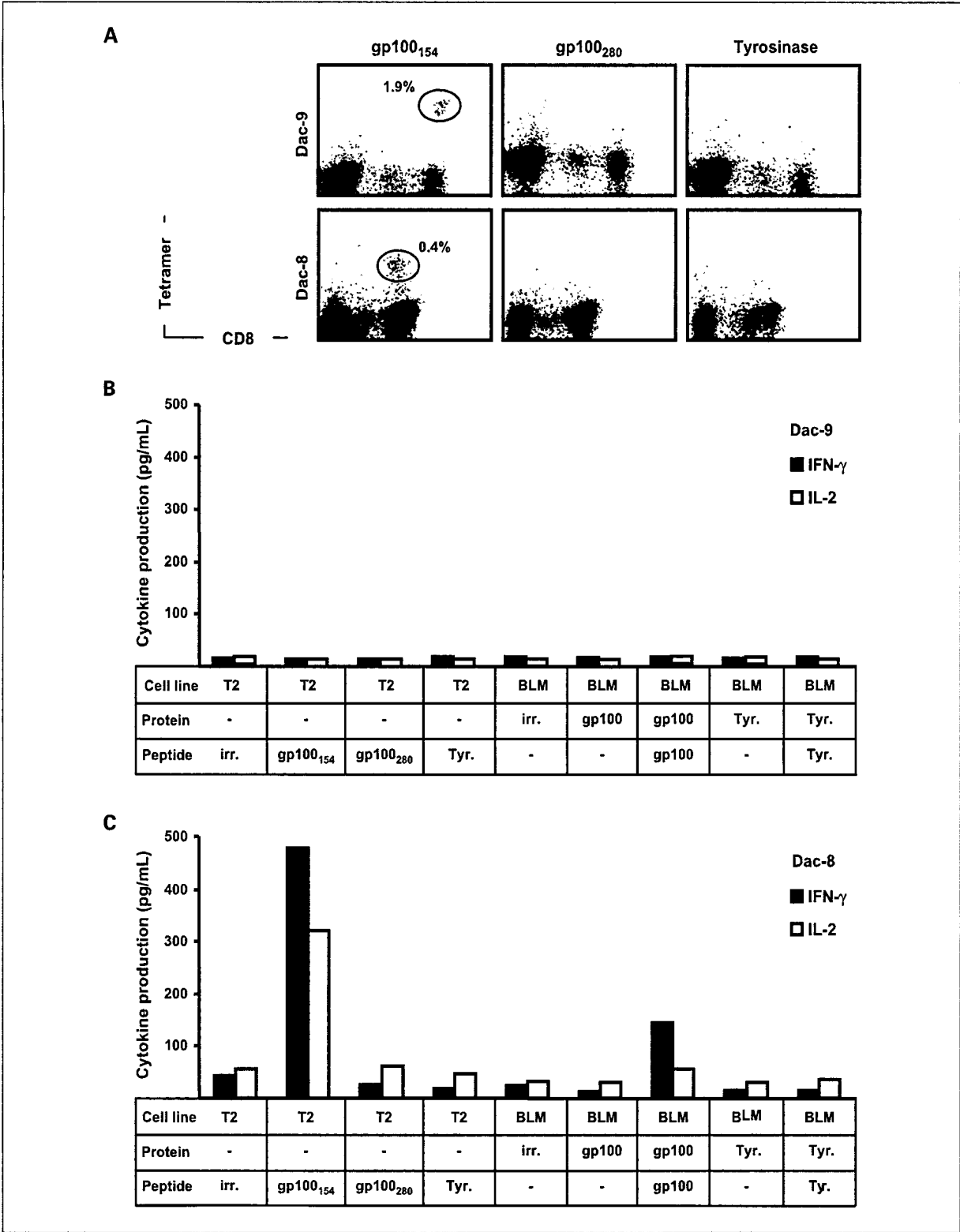


Fig. 4. Tumor-specific T cells *in vivo* in patients pretreated with daclizumab. A, flow cytometric tetramer analysis of T cells derived after three dendritic cell vaccinations from biopsies of positive DTH reactions to dendritic cells pulsed with gp100 peptides. Percentage of tetramer-specific CD8 cells is indicated in the dot plot. IFN- γ (black bars) and IL-2 (white bars) production of T cells derived from DTH reactions from patient Dac-8 (this is one representative patient out of four; B) and patient dac-9 (C) after stimulation with nonspecific and specific stimuli.

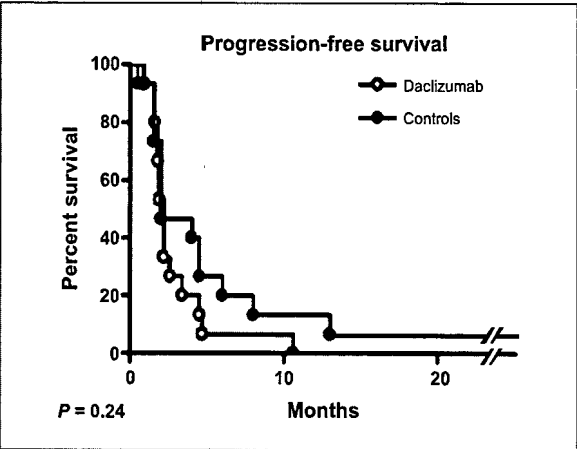


Fig. 5. Progression-free survival. Kaplan-Meier plot comparing the PFS of metastatic melanoma patients that were vaccinated with dendritic cells alone (●) and those that received dendritic cell vaccinations and pretreatment with daclizumab (○). No significant difference between both groups was detected ($P = 0.24$, log rank test).

study was to investigate whether a single low dose of daclizumab (0.5 mg/kg) transiently depletes Tregs in melanoma patients and to assess possible effects of this drug on immunity following dendritic cell vaccinations. In this proof-of-principle clinical trial we vaccinated 30 metastatic melanoma patients with peptide- and KLH-pulsed mature dendritic cells. Half of the patients were pretreated with daclizumab, a humanized antibody against CD25. High-dose daclizumab (1 mg/kg biweekly) was designed to target effector lymphocytes to prevent acute graft rejection following organ transplantation (41) and additionally proved effective for the treatment of patients with autoimmune diseases or T-cell leukemia (42). Beside activated lymphocytes, Tregs especially express high levels of CD25 and are highly dependent on IL-2 (25). IL-2 delivers signals that are essential for thymic development, peripheral survival, and functional fitness of Tregs (22–24). In mouse melanoma models it has been shown that transient depletion of Tregs using anti-CD25 monoclonal antibodies provokes tumor-specific immune responses (15, 18, 19). These observations have raised the question of whether interference with IL-2 signaling via daclizumab might decrease the number of functional Tregs (43).

We show that daclizumab efficiently depleted $CD4^+ FoxP3^+ CD25^{high}$ Tregs from the peripheral blood within 4 days after administration. Approximately 30 days after the administration, daclizumab was cleared from the circulation and $CD4^+ FoxP3^+ CD25^{high}$ Tregs reappeared. Immune response analysis revealed that significantly less patients pretreated with daclizumab developed functional vaccine-specific effector T cells and antibodies compared with controls. No antibodies against the “*de novo* antigen” KLH were present in the serum of daclizumab-treated patients. Subsequent *in vitro* analysis showed that in patients pretreated with daclizumab, vaccine-induced KLH-specific $CD4^+$ Th cells are generated and functional but only in the

absence of daclizumab. Vaccine-related tumor-associated antigen-specific $CD8^+$ T cells were detected in DTH biopsies from daclizumab-treated patients. However, these cells failed to acquire effector function upon target cell recognition even in the absence of daclizumab *in vitro*. These immunomonitoring data suggest that in daclizumab-treated patients the lack of proper Th-cell activation may have prevented effective generation of KLH reactive antibodies and vaccine-induced $CD8^+$ T-cell responses, both of which depend on effective T-cell help. The direct impact of daclizumab on $CD25^+$ B cells remains unclear.

Multiple explanations for why daclizumab did not enhance or might even inhibit the immunostimulatory efficacy of subsequent dendritic cell vaccines can be envisaged. Firstly, daclizumab does not deplete all $FoxP3^+$ cells. The residual $CD4^+ FoxP3^+ CD25^{intermediate}$ cells likely represent a functional (precursor) pool of Tregs. In addition, we cannot exclude that daclizumab-mediated $CD4^+ FoxP3^+ CD25^{high}$ -Treg depletion was less effective at the side of the tumor and tumor draining lymph nodes. Secondly, daclizumab recognizes CD25 that is not exclusively expressed on Tregs (44). CD25 is known to be expressed on activated $CD4$ and $CD8$ T cells, but melanoma cells themselves can also express low levels of CD25 (45, 46). Moreover, a single dose of daclizumab administered to melanoma patients transiently increases the number of regulatory NK cells that may have further compromised the efficacy of the subsequent dendritic cell vaccinations. Our *in vitro* data show that daclizumab in the serum of patients disarms effector T cells. Aside from inhibiting the *de novo* induction of effector T cells, daclizumab may also affect existing antitumor immune responses already present in the patients. Previously, it had been reported that the number of antigen-experienced $CD25^+$ lymphocytes is greatly elevated in tumors and their draining lymph nodes (13). Because we show that daclizumab depletes all $CD25^{high}$ lymphocytes *in vivo*, daclizumab may partly neutralize this pre-existing antitumor immune response. A similar hypothesis was raised by Curtin et al. to explain why anti-CD25 mAb in a mouse glioblastoma model can eliminate newly induced tumors but cannot inhibit tumor progression in established tumors (47). Thirdly, in all patients that received a single dose of 0.5 mg/kg daclizumab, the drug was still present during subsequent dendritic cell vaccinations. Residual daclizumab titers may have prevented the induction of functional $CD25^+$ effector cells by antigen-loaded dendritic cells. This may also explain why depletion of Tregs by Ontak can enhance T-cell responses upon vaccination in certain cancer patients (48). The half-life of this compound is very short, therefore the drug is no longer present at the time that vaccine-induced effector T-cell expansion occurs. In this context, it might be interesting to explore the original mouse mAb anti-Tac on which humanized daclizumab is based (49), as it will have a much shorter half-life than daclizumab in humans.

Our results indicate that immunotherapeutic trials in which antibodies are used to target CD25, or related Treg-depleting strategies such as CD25-directed toxins

(27–29, 50), must carefully monitor the effects on all CD25⁺, not Tregs only, and adjust timing and dosing schedules accordingly. A Treg-specific cell surface marker that could be targeted with antibodies to specifically deplete/inactivate Tregs would overcome the daclizumab-related problems encountered in this clinical trial.

We conclude that, although daclizumab depleted the CD4⁺FoxP3⁺CD25^{high} Tregs from the peripheral circulation, it did not enhance the efficacy of the dendritic cell vaccine. We show that residual daclizumab titers suppressed *de novo* induction of CD25⁺ effector cells during dendritic cell vaccinations. Our results indicate that for immunotherapeutic benefit of the transient Treg depletion, both timing and dosing of daclizumab administration are extremely important. Alternatively, to overcome the immunoregulatory influence of Tregs, novel approaches to specifically target and comprehensively eliminate or abrogate the function of Tregs *in vivo* are required.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Impact of Basiliximab on the Proportion of Regulatory T Cells and Their Subsets Early After Renal Transplantation: A Preliminary Report

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ABSTRACT

Object. This study aims to investigate the impact of anti-CD25 monoclonal antibody (basiliximab) on peripheral blood regulatory T cells (Treg) and their subsets in recipients early after renal transplantation.

Methods. Ten renal transplant recipients from November 2009 to February 2011 were divided into an induction therapy and a no-induction therapy group, mainly based on their will to accept basiliximab induction. Peripheral blood samples were collected at 2 hours before as well as 1 and 2 weeks after transplantation. Flow cytometry was used to analyze the proportion of regulatory T cells and their subsets.

Results. Compared with the no-induction therapy group, the proportions of both CD25^{high} T cell and CD25^{high} Foxp3⁺ double marked T cells were significantly decreased among in the basiliximab induction group remaining at low at 2 weeks. The subsets of activated Treg and cytokine-secreting Treg were also temporarily downregulated at 1 week after transplantation using basiliximab induction. However, the inhibitory transcriptional factor Foxp3 was not significantly affected by the induction therapy.

Conclusions. Anti-CD25 monoclonal antibody downregulated the proportion of regulatory T cell and its activated subsets in peripheral blood in the early stage after renal transplantation while the inhibitory function may still be spared.

MOST regulatory T cells (Treg) are CD4-positive and characterized by expression of CD25 and the transcription factor forkhead box P3 (Foxp3), which is required for both their development and function. Tregs seem to play an important role during induction and maintenance of renal allograft tolerance.^{1,2} Human Tregs are composed of three distinct subpopulations by phenotypic and functional features. The CD45RA⁺FoxP3^{low} resting Treg cell (rTreg) and CD45RA⁻FoxP3^{high} activated Treg cell (aTreg) are both suppressive in vitro, whereas the CD45RA⁻FoxP3^{low} cytokine-secreting T cell (cTreg) has no suppressive role in vitro but secretes a variety of pro-inflammatory cytokines like interleukin (IL)-17 interferon, etc.³

IL-2, a major T cell growth factor, mediates downstream signaling via its receptor, leading to T-cell activation and proliferation. Three protein chains (α , β , and γ) are noncovalently associated to form the IL-2 receptor (IL-2R). The α and β chains bind IL-2, while signal transduction following cytokine interaction is carried out by the γ -chain, along with the β subunit. Basiliximab,⁴ a chimeric mouse-human monoclonal antibody to the α

chain (CD25) of the IL-2R of T cells, has been used to prevent rejection in organ transplantation, especially in kidney transplantation. It acts as an antagonist at the IL-2 binding site of the p55 subunit (Tac antigen) of the

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high affinity IL-2R (CD25) on the surface of the activated T lymphocytes. However, the effects of basiliximab on Tregs, which also expresses CD25, are the subject of this study, that explores its impact on peripheral Tregs and their subsets early after renal transplantation.

METHODS

Baseline Characteristics of the Patients

From November 2009 to February 2011, we performed transplantations on 10 adult patients, aged 31 to 60 years. Six patients did receive basiliximab induction therapy (4/2, M/F), whereas the other four did not (2/2, M/F). The basiliximab (20 mg) was administered intravenously before and 4 days after transplantation. All patients received Cyclosporine or tacrolimus plus mycophenolate mofetil plus prednisone. Other key inclusion criteria were patients with panel reactive antibodies < 20% on the day of transplantation and those with previous and first renal transplantations. Patients were excluded if they had undergone a multiorgan transplantation, had previous long-term immunosuppression, were experiencing a generalized infection at the time of transplantation, had a history of malignancy, or were positive for HIV, hepatitis C virus antibody of hepatitis B surface antigen.

Preparation of Peripheral Blood Mononuclear Cells

Fresh heparinized peripheral blood samples from the 10 patients were collected at 2 hours pretransplantation as well as 1 and 2 week(s) posttransplantation. Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) density gradient centrifugation.

Flow Cytometry

Flow cytometry was performed using the BD fluorescent-activated cell sorter (FACS) system (BD FACS, Calibur, CO), acquiring 1 × 10⁶ cells for analysis using the Flowjo v5.7 software (Tree Star, Inc, OR). Cells were surface-stained with CD4-FITC, CD25-APC and CD45RA-PERCP CY5.5 and subsequently intracellular-stained with the PE-Foxp3 staining kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Statistics

IBM SPSS 19.0 (International Business Machines Corp, New York, NY) was used for data analysis. Student *t* test was used to compare the frequency of cell subgroups for the basiliximab induction versus noninduction cohorts. Overall, a *P* value ≤ .05 was regarded as significant.

RESULTS

Baseline Data in Basiliximab Induction and No-induction Groups

There was no significant difference in mean age, gender, or serum creatinine values at 2 weeks after renal transplantation between the groups (Table 1).

Comparison of Treg and its Subgroups in Peripheral Blood Between Basiliximab Induction and No-induction Groups

The percentage of CD4⁺CD25^{high} T cells in peripheral blood was decreased significantly by the basiliximab at 1 week posttransplantation compared with the no-induction group (0.57 ± 0.17 versus 9.61 ± 2.90, *P* < .05). The low level was maintained to 2 weeks posttransplantation (0.42 ± 0.11 versus 7.20 ± 0.99, *P* < .05). Similarly, the percentage of CD4⁺CD25^{high}Foxp3⁺ regulatory T cells in peripheral were also significantly reduced by basiliximab compared with the no-induction group (0.05 ± 0.03 versus 7.51 ± 2.0, *P* < .05) at 1 (0.08 ± 0.05 versus 7.08 ± 0.78, *P* < .05) and at 2 weeks, respectively. Moreover, the percentages of CD45RA⁺Foxp3^{low} aTreg and CD45RA⁺Foxp3^{low} cTreg cell were reduced among the basiliximab treated group at 1 week but increased in the 2nd week, after transplantation while the no-induction group showed a reverse tendency. However, the inhibitory transcriptional factor Foxp3 showed no significant difference between the two groups (Table 2).

DISCUSSION

We compared the proportion of Tregs and their subsets in the peripheral blood of renal transplant recipients with versus without basiliximab induction. Patients who underwent induction therapy displayed significantly lower levels of CD25^{high} T cells and CD25^{high}Foxp3⁺ conventional regulatory T cells. In contrast to the no-induction group, basiliximab also decreased the Treg subsets, including CD45RA⁺Foxp3^{low} aTreg and CD45RA⁺Foxp3^{low} cTreg at 1 week posttransplantation. However, the inhibitory transcriptional factor Foxp3 showed no significant difference between these two groups.

To date, dominant peripheral tolerance in renal transplantation has been mostly associated with regulatory T cells. The intracellular expression of the transcription factor Foxp3 has been considered to be the best marker of Treg. Foxp3 is essential for the development, function

Table 1. Baseline Data in the Basiliximab Induction and No-induction Groups

Data	Induction Group	No-induction Group	
Age (y ± SEM)	41.50 ± 4.17	44.76 ± 6.95	<i>P</i> > 0.05
Gender (M/F)	4/2	2/2	<i>P</i> > 0.05
SCr at 2nd week after operation (μmol/L)	91.33 ± 10.77	112.00 ± 11.75	<i>P</i> > 0.05
Immunosuppression therapy	CNI + MMF + Pred	CNI + MMF + Pred	

Abbreviations: SEM, standard error of mean; M, male; F, female; SCr, serum creatinine level; CNI, cyclosporin A or tacrolimus; MMF, mycophenolate mofetil; Pred, prednisolone.
Chi-square test was used for comparison of gender.
Student *t* test was used for age and SCr between the two groups.

Table 2. Comparison of Treg and its Subsets in Peripheral Blood Between the Basiliximab Induction and No-induction Groups (%)

Cell Subsets	Groups	Pretransplant	Post-transplant	
			1st week	2nd week
CD4 ⁺ CD25 ^{high}	Induction	5.51 ± 0.70	0.57 ± 0.17	0.42 ± 0.11
	No-induction	4.45 ± 0.62	9.61 ± 2.90	7.20 ± 0.99
		P = .945	P = .004	P = .000
CD4 ⁺ Foxp3 ⁺	Induction	6.39 ± 0.59	5.21 ± 0.57	8.05 ± 1.00
	No-induction	6.28 ± 0.91	8.72 ± 2.05	8.10 ± 1.30
		P = .846	P = .082	P = .979
CD4 ⁺ CD25 ^{high} Foxp3 ⁺ Treg	Induction	4.74 ± 0.74	0.05 ± 0.03	0.08 ± 0.05
	No-induction	4.92 ± 0.64	7.51 ± 2.0	7.08 ± 0.78
		P = .781	P = .001	P = .000
Cytokine secreting Treg	Induction	4.01 ± 0.64	3.73 ± 0.42	5.41 ± 1.04
	No-induction	3.22 ± 0.34	5.68 ± 1.26	4.72 ± 0.74
		P = .784	P = .121	P = .944
Activated Treg	Induction	0.96 ± 0.29	0.47 ± 0.20	0.54 ± 0.15
	No-induction	0.66 ± 0.20	1.27 ± 0.48	0.78 ± 0.07
		P = .710	P = .110	P = .359

Student t test was performed for comparison of Treg and its subsets between the two groups.

and survival of Treg. Mutations or polymorphisms in the genes encoding these molecules may predispose to autoimmune or lymphoproliferative disease.^{5,6} Furthermore, the specific role of Foxp3 in the function of nTregs was highlighted by the fact that retroviral transduction of Foxp3 into CD4⁺CD25[−] T cells could convert them to functional Tregs able to suppress proliferation of other T cells *in vitro* and to inhibit development of autoimmune diseases mediated by effector T cells *in vivo*.^{7,8} Interestingly, recent work has reported three functional subsets of Treg based on biomarkers: CD45RA⁺Foxp3^{low} resting Treg (rTreg), CD45RA[−]Foxp3^{high} activated Treg (aTreg), and CD45RA[−]Foxp3^{low} cytokine secreting Treg (cTreg). aTreg show immunosuppressive functions, while cTreg actually prompt an immune response by secreting pro-inflammatory cytokines. Both aTreg and cTreg are activated Treg, while rTreg are resting aTreg.³

Anti-CD25 monoclonal antibody has been applied to induction therapy in renal transplantation for years. It specifically antagonizes the α-chain of the IL-2R (CD25) on the T cell surface inhibiting T cell activation and proliferation.⁹ Thus basiliximab may affect regulatory Tcells and their subsets. In this study, we showed that basiliximab significantly decreased the percentage of CD4⁺CD25^{high} T cells in the early stage after renal transplantation, indicating effective blockade of CD25. Moreover, the proportion of CD25^{high}Foxp3⁺ Treg was also significantly reduced by basiliximab but without any significant difference in Foxp3 expression. Based on these findings, we may assume that although the basiliximab does reduce Treg quantitatively, it may not affect the Foxp3-defined immunosuppressive role of Treg.

As activated subgroups, the cTreg and aTreg may participate directly in posttransplanted immune balance. cTregs secrete pro-inflammatory cytokines such as IL-17 that promote rejection whereas the aTregs antagonize the role of cTreg, leading to a stable immune system.¹⁰ In

this study, we first investigated the short-term influence of basiliximab treatment on peripheral blood Treg subsets showing decrease in synchronous cTreg and aTreg at 1 week after basiliximab induction therapy with increases in the no-induction group. This finding may indicate a low-response balance in the early stage after administration of the basiliximab, during which the activity of immune system is switched into a low response level due to the lack of activated subsets of T cells. Moreover, the parallel decrease of aTreg may also be attributed to the low immune activity when there is no need to rapidly produce a large number of aTreg from resting Treg.

In conclusion, this study showed basiliximab induction therapy to downregulate Tregs and their active subsets in the early stage after renal transplantation. It had no effect on Foxp3 expression. Therefore, this short period change in the immune system may promote a low-response homeostasis which is important to reset the immune balance and prevent acute rejection.

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