

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I		
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 18		
ACT: 519 PRESENTACION			

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
LUZ CLEMENCIA SUAREZ DE PAEZ

Asunto: Radicación: 11-50626- -10
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 13235
 Folios: 18

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/EP2009/61210				
Fecha de Presentación Internacional	31/08/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No11-50626-00000-0000	26/Abril/2011
Reivindicaciones:	Radicado No11-050626-00000-0000	26/Abril/2011
Dibujos:	Radicado No11-050626-00000-0000	26/Abril/2011
Oposiciones:	Radicado No11-050626-00000-0000	Lafrancol 18/Abril /2012
Respuesta del solicitante:	Radicado No11-050626-00009-0000	9/Noviembre/2012
Prioridad:	GB 0817810.5	29/Septiembre/2008
	GB 0817811.3	29/Septiembre/2008
	GB 0817809.7	29/Septiembre/2008
	EP 2009 052811	10/Marzo/2009
	EP 2009 052809	10/Marzo/2009
	EP2009 052810	10/Marzo/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

En el presente caso el número de reivindicaciones que se estudiaran es estudiadas es de 1 a 83. El solicitante anexa pago de reivindicaciones adicionales.

Título de la solicitud: “Composición para tratar enfermedad”

El problema planteado en esta solicitud consiste en la necesidad de proporcionar, una composición farmacéutica útil para tratar inflamación, osteoartritis, artritis reumatoide. Para resolver este problema técnico la solicitud en estudio proporciona una composición que comprende un anticuerpo antiCD4 y metotrexalato

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 039/395.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 21-56 y 78-82 no es patentable de acuerdo con el Art citado.

No obstante las reivindicaciones 11-20, 58, 60 y 64 se encabezan como composición o agente, éstas se definen en términos de su administración a un sujeto, las dosis de administración y la administración simultánea, por separado o en forma secuencial de diferentes agentes, por lo tanto no son patentables de acuerdo con el Art citado.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 59, 62, 63 y 66-82 no es patentable, de acuerdo con el Art 14. Las reivindicaciones 57, 61 y 65 que se encabezan como composición o agente, se definen en términos de su uso, por lo tanto no son patentables de acuerdo con el Art citado.

6. De la solicitud de patente

7. Título

El título debe ser más concreto con el objeto de la solicitud, por lo cual se sugiere al solicitante hacer la corrección necesaria.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 – 10 no son claras porque:

- 1- La reivindicación 1 se define en términos de resultado a alcanzar, se debe definir estructuralmente la composición.
- 2- La reivindicación 2 se refiere a “un equipo” sin definirlo por sus características técnicas, por lo cual no permite distinguir la invención del estado de la técnica. Adicionalmente no se encuentra soporte descriptivo de la obtención de un equipo por lo tanto se sugiere a la

solicitante eliminar esta reivindicación del alcance del pliego reivindicatorio.

- 3- La vía de administración de la composición no es una característica técnica de la misma. Se sugiere eliminarla de las reivindicaciones 1, 3-20.
- 4- El anticuerpo de la reivindicación 1-9 debe definirse en términos de su estructura completa, mencionando las secuencias que lo conforman en la cadena ligera y pesada.
- 5- En las reivindicaciones 6 y 9 la referencia a fragmentos, derivados o variaciones del anticuerpo no es clara porque no permite definir una secuencia exacta definida y probada. Se sugiere eliminar referencia a fragmentos y derivados.
- 6- En la reivindicación 10 se definen las secuencias del polipéptido indicando el porcentaje de identidad, lo que no permite establecer la secuencia aminoácida exacta a la que se refiere. Se sugiere eliminar toda referencia a porcentajes de identidad.
- 7- No se encuentra sustento en la descripción de la obtención o prueba de una composición o un equipo como los reclamados en la solicitud.

Descripción (Art 28 D 486)

La descripción no divulga la invención de manera suficientemente completa para que el la persona del oficio normalmente versada en la materia pueda ejecutarla ó reproducirla. El eje central de la solicitud es un método de tratamiento y no el desarrollo u obtención de una composición o un equipo.

8. Análisis de la Oposición

Argumentaciones de Lafranco

Dice el opositor que esta solicitud pretende obtener patente para 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 que son prácticas y eventos conocidos en la industria farmacéutica.

Las composiciones farmacéuticas de la solicitud involucran agentes tales como Daclizumab y Basiliximab que son moléculas descubiertas y plenamente identificadas. Para lo cual el opositor cita las fichas técnicas de The Merck Index. "An encyclopedia of chemicals, drugs, and biologicals. Fourteenth Edition. 2006. Página 472.(DACLIZUMAB) y The Merck Index. "An encyclopedia of chemicals, drugs, and biologicals. Fourteenth Edition. 2006. Página 167. (BASILIXIMAB). El solicitante adjunta dos páginas de pruebas (folios 130-131).

De igual manera el opositor menciona que la molécula daclizumab obtuvo derecho patentario concedido por Estados Unidos de América No US 5530101 (1996) y basiliximab obtuvo derecho patentario concedido por la oficina Europea de Patentes, No EP 449769 (1991).

Respuestas del Solicitante

El solicitante argumenta respecto a la oposición para novedad que el apoderado de la oposición no demostró que la invención ni los conocimientos técnicos que de ella se desprenden hayan sido accesibles al público. Para el solicitante la presente solicitud describe como los agentes más adecuados a los anticuerpos con uno más dominios variables que son capaces de enlazarse a CD 4 que es un objetivo diferente al descrito para daclizumab y basiliximab, para lo cual aporta los siguientes documentos: Joanes 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 metastásico, 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 (2010). El solicitante también argumenta que adicionalmente, es de anotar que ni la presente US 5530101 ni la US EP 0449769 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. Para el apoderado el anterior

análisis representa una clara y sustancial diferencia entre la invención solicitada y los documentos del estado de la técnica y lo revelado en los documentos citados como fundamento de la oposición. Respecto a nivel inventivo el solicitante argumenta que el apoderado de la sociedad opositora se limita simplemente a listar una serie de documentos en que se divulgan algunas moléculas, no es idóneo ni convincente para demostrar la ausencia de nivel inventivo de la patente solicitada.

Respuesta del Examinador Técnico

El documento presentado en la oposición no desvirtúa la novedad y/o nivel inventivo del objeto de esta solicitud; puesto que no divulga una composición farmacéutica que comprenda un anticuerpo antiCD4+CD25+ y metotrexato que son las características técnicas esenciales de la invención y no se debe considerar la capacidad de activar células t regulatorias CD4+CD25+ que es un resultado a alcanzar para desvirtuar la novedad y/o nivel inventivo del capítulo reivindicatorio de la presente solicitud.

Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: septiembre 29 de 2008. y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	WO 2004 083247	“Humanized anti-CD4 antibody with immunosuppressive properties”	30/Septiembre/2004
D2	Journal of experimental medicine. (Vol. 200(3). Págs 277-285)	“Compromised function of regulatory T cells in reumatoide arthritis and reversal by anti-TNF alpha therapy”	02/Agosto/2004

El documento D1¹ divulga un anticuerpo humanizado anti CD4 y su uso en inmunomodulación. (Pág. 1, líneas 1 – 2).

El documento D2² divulga un tratamiento con (anti TNF□; Infliximab) que restaura la capacidad de las células T para inhibir la producción de citosinas y para transmitir un fenotipo supresor.(Resumen).

9 . Examen de Patentabilidad (Art 14 D486)

Nivel inventivo (Art18 D486)

La reivindicación 1 consiste en *“una composición farmacéutica que comprende un agente capaz de activar células T regulatorias CD4+CD25+ y metotrexato”*.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Composición farmacéutica que comprende:	Composición que contiene	Combinación que comprende:
anticuerpo antiCD4+CD25+	Anticuerpo anti -CD4 B-F5. (resumen)	Anticuerpo anti-TNF□ (Pág 278)
Metotrexato	No menciona	Metotrexato (Pág 278)

11http://worldwide.espacenet.com/maximizedOriginalDocument?flavour=maximizedPlainPage&locale=en_EP&FT=D&date=20040930&CC=WO&NR=2004083247A1&KC=A1

2 <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2211983/#bib29>

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una composición inmunosupresora que contiene anticuerpo anti CD4 B-F5 (resumen) y revela anticuerpo anti CD4 hB-F5 H37L/L4M SEC.ID N°1 y SEC ID N°2. (Fig 3-4) .

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga una combinación que contiene Anticuerpo anti-TNF α (Pág 278) y Metotrexato (Pág 278)

La diferencia entre la invención, definida en la reivindicación 1 y la composición de D1 consiste en que la composición farmacéutica además del anticuerpo anti CD4 contiene metotrexato, para emplearse en tratamiento de pacientes con artrosis reumatoide

La diferencia entre la invención definida en la reivindicación 1 y el documento D2 es que el anticuerpo y el metorexato se encuentra presentes en una composición y no en una combinación.

No se evidencia un efecto técnico mejorado en la actividad de la composición en la que se incluye metotrexato, para un tratamiento de paciente con enfermedad de artrosis reumatoide.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar una composición inmunosopresora que contiene anticuerpo anti-CD4 B-F5 para terapia en tratamiento en pacientes con artrosis reumatoide, procedimiento conocido en D1, agregando a la composición un agente activo.

Sin embargo, la utilización de un agente activo como metotrexato en combinación con un anticuerpo anti-TNF α (infliximab) para tratamiento en pacientes con artrosis reumatoide ya está divulgada en el documento D2. (Pág 278)

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría metotrexona del documento D2 en el procedimiento divulgado en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 2-10 no mencionan características adicionales, se considera que tales reivindicaciones tampoco tienen nivel inventivo

Conclusión:

Las reivindicaciones 1 a 10 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

10. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO 2004 083247	1 – 10	Nivel inventivo
D2	Journal of experimental medicine	1 – 10	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-07-30

Elaboró: Edna Milena Bautista Rodriguez
Revisó: Jeny Paola Villate Becerra

Compromised Function of Regulatory T Cells in Rheumatoid Arthritis and Reversal by Anti-TNF α Therapy

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Abstract

Regulatory T cells have been clearly implicated in the control of disease in murine models of autoimmunity. The paucity of data regarding the role of these lymphocytes in human autoimmune disease has prompted us to examine their function in patients with rheumatoid arthritis (RA). Regulatory (CD4⁺CD25⁺) T cells isolated from patients with active RA displayed an anergic phenotype upon stimulation with anti-CD3 and anti-CD28 antibodies, and suppressed the proliferation of effector T cells in vitro. However, they were unable to suppress proinflammatory cytokine secretion from activated T cells and monocytes, or to convey a suppressive phenotype to effector CD4⁺CD25⁻ T cells. Treatment with antitumor necrosis factor α (TNF α ; Infliximab) restored the capacity of regulatory T cells to inhibit cytokine production and to convey a suppressive phenotype to "conventional" T cells. Furthermore, anti-TNF α treatment led to a significant rise in the number of peripheral blood regulatory T cells in RA patients responding to this treatment, which correlated with a reduction in C reactive protein. These data are the first to demonstrate that regulatory T cells are functionally compromised in RA, and indicate that modulation of regulatory T cells by anti-TNF α therapy may be a further mechanism by which this disease is ameliorated.

Key words: T lymphocytes • tolerane • autoimmune disease • TNF α • cytokines

Introduction

Studies in rodents have provided firm evidence for the existence of a naturally occurring population of CD4⁺CD25⁺ professional regulatory/suppressor T cells, which, upon in vitro TCR-mediated stimulation, suppress the proliferation of effector T cells (1, 2). These cells appear central to the control of T cell homeostasis and in the modulation of immune responses to autoantigens, cancer cells, pathogens, and alloantigens (1, 2). In the periphery of young mice not prone to autoimmune diseases, regulatory T cells constitute a stable 10% of CD4⁺ T cells. This proportion appears to be reduced in mice genetically prone to autoimmune disease such as diabetes (3, 4). Transfer of regulatory T cells is able to prevent a wide range of experimental autoimmune diseases, including diabetes, experimental autoimmune encephalo-

myelitis, and colitis (3–6). In addition, depletion of regulatory T cells has been shown to exacerbate various experimental autoimmune diseases, including collagen induced arthritis (7). In humans, an analogous population of CD4⁺CD25⁺ regulatory T cells has been identified in the peripheral blood (PB; 8–11) and in the thymus (12, 13). In comparison to the wealth of evidence from animal models, much less is known about human regulatory T cells and their potential to suppress autoimmune disease in patients. One recent analysis of human regulatory T cells demonstrated a reduction in their numbers in the PB of newly diagnosed type-1 diabetic patients, suggesting that a deficiency in this subset could be responsible for the breakdown of tolerance in human diabetes (14).

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that ultimately leads to the destruction of joint architecture. The pathogenic events that lead to the development of RA are not fully understood, although the pivotal role

The online version of this article contains supplemental material.

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Abbreviations used in this paper: CBA, cytometric bead array; PB, peripheral blood; RA, rheumatoid arthritis.

that proinflammatory cytokines (i.e., such as TNF α , IL-1 β , IL-6, etc.) play in the induction and maintenance of this disease is well documented (15). Moreover, these cytokines are important in the homeostasis of T lymphocytes (16), including regulation of the CD4⁺CD25⁺ regulatory T cell subset. In RA, the CD4⁺CD25⁺ regulatory T cell subset represents ~5–10% of the CD4⁺ T cell population, and this population has been shown to suppress the in vitro proliferation of autologous CD4⁺ T cells (17). Because proinflammatory cytokines are critical to the pathogenesis of RA, we postulated that regulatory T cells in active RA, although still capable of suppressing T cell proliferation, may be defective in controlling proinflammatory cytokine production. Therefore, we set out to investigate any changes in the function and/or number of regulatory T cells in the PB of patients with active RA and to determine if clinical response to anti-TNF α therapy results in changes in this cell population.

In this paper, we present evidence to suggest that regulatory T cells derived from patients with active RA are defective in their ability to suppress cytokine production and in their ability to convey a suppressive phenotype to CD4⁺ effector T cells. We go on to demonstrate that clinical response to anti-TNF α , but not conventional therapies, in RA patients is correlated with an increased number of PB regulatory T cells, suggesting a possible additional mechanism by which blockade of TNF α results in amelioration of RA.

Materials and Methods

Study Population. 27 patients with active RA, fulfilling the revised classification criteria of the American College of Rheumatology for RA, were evaluated before and after anti-TNF α therapy (Infliximab was given at a dose of 3 mg/kg i.v. at weeks 0, 2, 6, and at every 8 wk in combination with stable doses of methotrexate 7.5–15 mg/wk orally) and stable nonsteroidal anti-inflammatory drugs. Patients taking prednisolone were excluded from the study because steroids are known to affect lymphocyte function and CD25 expression (18). Only patients with a disease activity score of >5.1 were treated with anti-TNF α therapy. Response was defined as a drop in the disease activity score of >1.2. Patients responding to conventional dosages of methotrexate (15–25 mg/wk) were also included in this analysis.

Antibodies. The following Abs were used: FITC-conjugated anti-CD4 (RPAT4), Cy-Chrome-conjugated anti-CD25 (M-A251), and PE-conjugated anti-CD3 (UCHT1). Staining of MACS[®]-sorted T cells included the following: PE-conjugated, FITC-conjugated anti-CD3 (UCHT1); anti-CD4 (RPAT4); anti-CD25 (4E3); anti-CD28 (CD28.2); and anti-CTLA-4 (BNI3; all obtained from BD Biosciences). For T cell activation the stimulatory Abs, anti-CD3 (OKT3) and anti-CD28 (CD28.2; obtained from BD Biosciences) were used as indicated.

Cell Isolation. PBMCs were isolated by Ficoll-Paque Plus (Amersham Biosciences) gradient centrifugation. Cells were cultured in RPMI 1640 media supplemented with 2 nM L-glutamine, 5 mM Hepes, 100 U/ μ g/ml penicillin/streptomycin, 0.5 mM sodium pyruvate, 0.05 mM of nonessential amino acids (both obtained from Life Technologies), and 10% FCS (all obtained from BioWhittaker) in 96-well U-bottom plates (Nunc).

Non-CD4⁺ T cells were stained using a biotin antibody cocktail (10 μ l/10⁷ total cells), incubated for 10 min at 4°C, magnetically labeled with antibiotin microbeads (20 μ l/10⁷ total cells), incubated for 15 min at 4°C, washed twice, and depleted over a MACS[®] LD column. CD4⁺CD25⁺ T cells were directly labeled with anti-CD25 microbeads (10 μ l/10⁷ CD4⁺ cells), incubated for 15 min at 4°C, and positively selected using MACS[®] MS columns according to manufacturer's instructions (Miltenyi Biotec). T cell subpopulations of CD4⁺CD25⁺ T cells were used immediately after isolation. All the plate-bound anti-CD3 cultures received 10⁴ irradiated PBMCs. In separate experiments, cells were isolated using a MoFlo cell sorter after staining using FITC-conjugated anti-CD4 and Cy-Chrome-conjugated anti-CD25. The purities of the populations obtained using these two methods of cell isolation are shown in Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20040165/DC1>.

Cytokine Detection. For analysis of intracellular cytokine production T cells were stimulated with either 5 μ g/ml of soluble anti-CD3/anti-CD28 or 1 μ g/ml of plate-bound anti-CD3/irradiated PBMCs for 48 h. 2 μ M monensin was added for the last 5 h. Staining of CD4 and CD25 surface expression was performed. Cells were washed, fixed, permeabilized, and stained for detection of intracellular cytokines using PE-conjugated anti-IFN γ , TNF α , or IL-10. Alternatively, supernatants were collected, before the addition of monensin, and IL-2, IL-4, IL-5, IL-10, IFN γ , and TNF α levels were measured by the cytometric bead array kit (CBA kit; BD Biosciences), according to the manufacturer's instructions.

Proliferation Assay. To assess proliferation of different CD4⁺ subtypes, 10⁵ cells sorted of MACS purified T cells were incubated in complete medium with 1 μ g/ml of plate-bound anti-CD3, 1 μ g/ml of plate-bound anti-CD3, and 5 μ g/ml of soluble anti-CD28 or 5 μ g/ml of soluble anti-CD3/anti-CD28 in 96-well U-bottom plates (Nunc). After 5 d of culture, [³H]Tdr was added for the remaining 8 h of culture. Proliferation was measured using a liquid scintillation counter.

Flow Cytometric Analysis. Immunofluorescence staining was performed after washing the cells twice with PBS plus 0.5% human serum albumin. Cells were incubated for 20 min at 4°C with each mAb (5 μ g/ml, 10⁵ cells/test). After washing with cold PBS/human serum albumin, the indirectly labeled cells were incubated with FITC- and PE-conjugated second-step mAb for 20 min at 4°C, washed three times, and analyzed by flow cytometry (FACScalibur[™], CELLQuest[™] software; Becton Dickinson).

Statistical Analysis. Analysis for statistically significant differences was performed with Student's *t* test. *p*-values <0.05 were considered significant.

Online Supplemental Material. Fig. S1 shows the purity of the populations isolated using either a MoFlo cell sorter or a MACS[®] column as analyzed by FACS[®]. The clinical details of the patients studied are included in Table S1. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20040165/DC1>.

Results

CD4⁺CD25⁺ T Cells Isolated from RA Patients Are Anergic and Suppress the Proliferation of Effector T Cells. To test the hypothesis that CD4⁺CD25⁺ regulatory T cells isolated from active RA patients may be functionally defective compared with those isolated from healthy individuals, or from the same patients after anti-TNF α treatment, CD4⁺CD25⁺

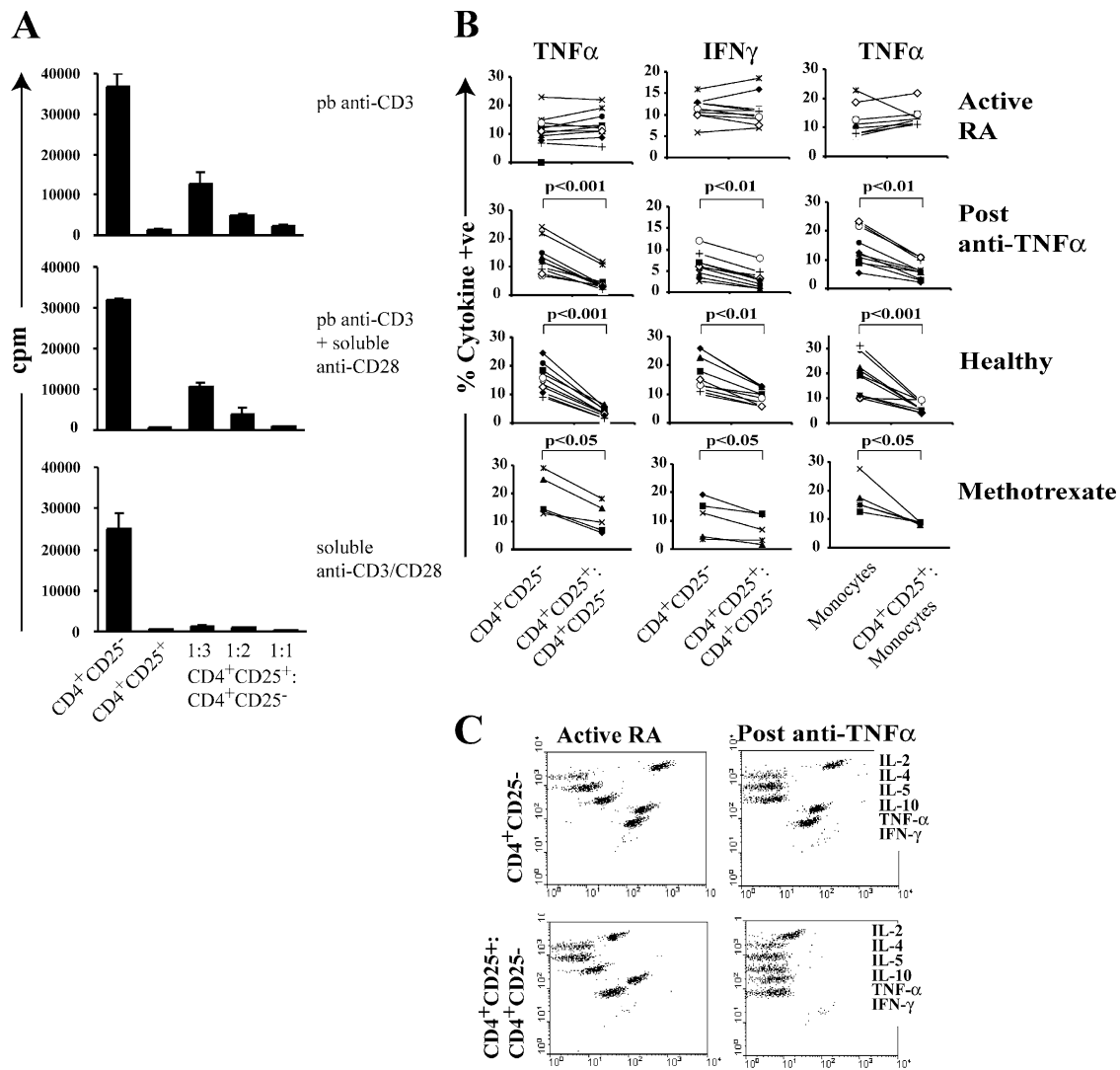


Figure 1. Regulatory T cells from the PB of patients with active RA can suppress proliferation but not proinflammatory cytokine production by effector T cells and monocytes. (A) PB was collected from patients with RA; MACS[®]-sorted CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells were cultured either alone or mixed at 1:3, 1:2, 1:1 ratio (10⁵ cells/well) and stimulated with 1 μg/ml of plate-bound anti-CD3, 1 μg/ml of plate-bound anti-CD3/5 μg/ml of soluble anti-CD28, and 5 μg/ml soluble anti-CD3/anti-CD28 for 48 h. Proliferation after 5 d was determined by [³H]Tdr incorporation. Results are expressed as mean ± SEM of triplicate cultures. One out of five independent experiments is shown. (B) MACS[®]-sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from 10 patients before and after anti-TNFα therapy (3 mo) with 5 patients responding to methotrexate therapy or 10 healthy controls were cultured alone or mixed at a 1:1 ratio (10⁵ cells/well) and stimulated with 5 μg/ml of soluble anti-CD3/anti-CD28 for 48 h. Monocytes were stimulated with 10 ng/ml LPS for 48 h. 2 μM monensin was added for the last 5 h of culture. Staining of CD4 and CD25 surface expression was performed and, subsequently, the cells were washed, permeabilized, and stained with PE-conjugated anti-TNFα or anti-IFNγ. (C) Cytokines from the supernatants collected from the experiment (B) were measured by CBA. 1 out of 10 experiments is illustrated.

T cells were purified by MACS[®] sorting (as described in Materials and Methods) and cultured alone or mixed with CD4⁺CD25⁻ T cells. Three different stimuli were used to reveal any possible defect in the CD4⁺CD25⁺ T cell population in the PB of patients with RA. The results in Fig. 1 A show that CD4⁺CD25⁺ T cells isolated from active RA patients displayed an anergic phenotype and were able to inhibit the proliferation of effector CD4⁺CD25⁻ T cells in a dose-dependent mode when different T cell receptor signal strengths were used. CD4⁺CD25⁺ T cells isolated from active RA patients were capable of suppressing the proliferation of responder T cells to a similar level as found in

healthy individuals or in the same group of patients 3 mo after anti-TNFα treatment (unpublished data).

CD4⁺CD25⁺ Regulatory T Cells Isolated from RA Patients Do Not Suppress Cytokines Produced by Effector T Cells. As proinflammatory cytokines play an essential role in the pathogenesis of RA, next we assessed whether CD4⁺CD25⁺ regulatory T cells, isolated before and after anti-TNFα treatment, were able to suppress proinflammatory cytokine production by effector T cells. Similar to the suppressive effects that regulatory T cells have on the proliferation of effector T cells, the high levels of TNFα and IFNγ synthesized by the responder CD4⁺ T cells were

suppressed after coculture with $CD4^+CD25^+$ T cells derived from healthy or anti-TNF α -treated patients (Fig. 1 B). However, regulatory T cells isolated from patients with active RA failed to inhibit the production of these cytokines by $CD4^+CD25^-$ effector T cells. Similarly, TNF α production by monocytes, a key producer of this cytokine in RA, was also markedly inhibited by $CD4^+CD25^+$ T cells isolated from healthy individuals and patients with RA after treatment. No IL-10 production was detected in any of the samples analyzed (unpublished data). The response of $CD4^+CD25^+$ T cells isolated from patients responding to methotrexate treatment was included in this analysis. Although $CD4^+CD25^+$ T cells efficiently suppressed TNF α produced by monocytes, the ability to inhibit cytokines produced by effector T cells was less effective compared with $CD4^+CD25^+$ T cells isolated from anti-TNF α -treated patients (20–30% inhibition compared with 60–70% inhibition after anti-TNF α). To complement the results obtained by intracellular staining, cytokine production was also measured in the supernatants derived from the aforementioned experiments by CBA. In keeping with the results obtained from the intracellular staining, only regulatory T cells isolated from anti-TNF α -treated patients strongly suppressed TNF α and IFN γ released by effector $CD4^+CD25^-$ T cells upon anti-CD3/CD28 stimulation *in vitro*. A reduction in IL-2 production was detected in both groups. Comparable results were obtained when plate-bound anti-CD3 and irradiated PBMCs were used instead of soluble stimuli (unpublished data).

$CD4^+CD25^{high}$ T Cells from Active RA Patients Are Functionally Defective. The results described thus far are based on the use of populations of $CD4^+CD25^+$ T cells purified with magnetic beads, containing mixtures of $CD25^{high}$ and $CD25^{low}$ cells. It has been shown previously that amongst $CD4^+$ T cells expressing CD25, only those expressing high levels of CD25 display regulatory function (10). To dissect which subset of $CD4^+CD25^+$ T cells (high or low) was responsible for the suppressive effect on cytokines released by the effector T cells, $CD4^+$ T cells were sorted according to their CD25 expression (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20040165/DC1>). Results in Fig. 2 A demonstrate that the suppressive effect on cytokine production resides in the $CD4^+CD25^{high}$ T cell fraction when isolated from anti-TNF α -treated patients. In contrast, the $CD4^+CD25^{high}$ T cells from patients with active RA were unable to suppress TNF α and IFN γ produced by $CD4^+CD25^-$ T cells. No differences were found in TNF α or IFN γ produced by $CD4^+CD25^{high}$ T cells isolated from patients before or after anti-TNF α treatment. Cytokines were also measured in the culture supernatants by CBA, and the results in Fig. 2 B reproduce the aforementioned findings. In agreement with the suppression of proliferation results shown in Fig. 1 A, regulatory T cells irrespective of their provenance strongly suppressed the production of IL-2 released by autologous effector T cells. No consistent changes in IL-5, IL-4, and IL-10 were found in the different samples analyzed (unpublished data). Together,

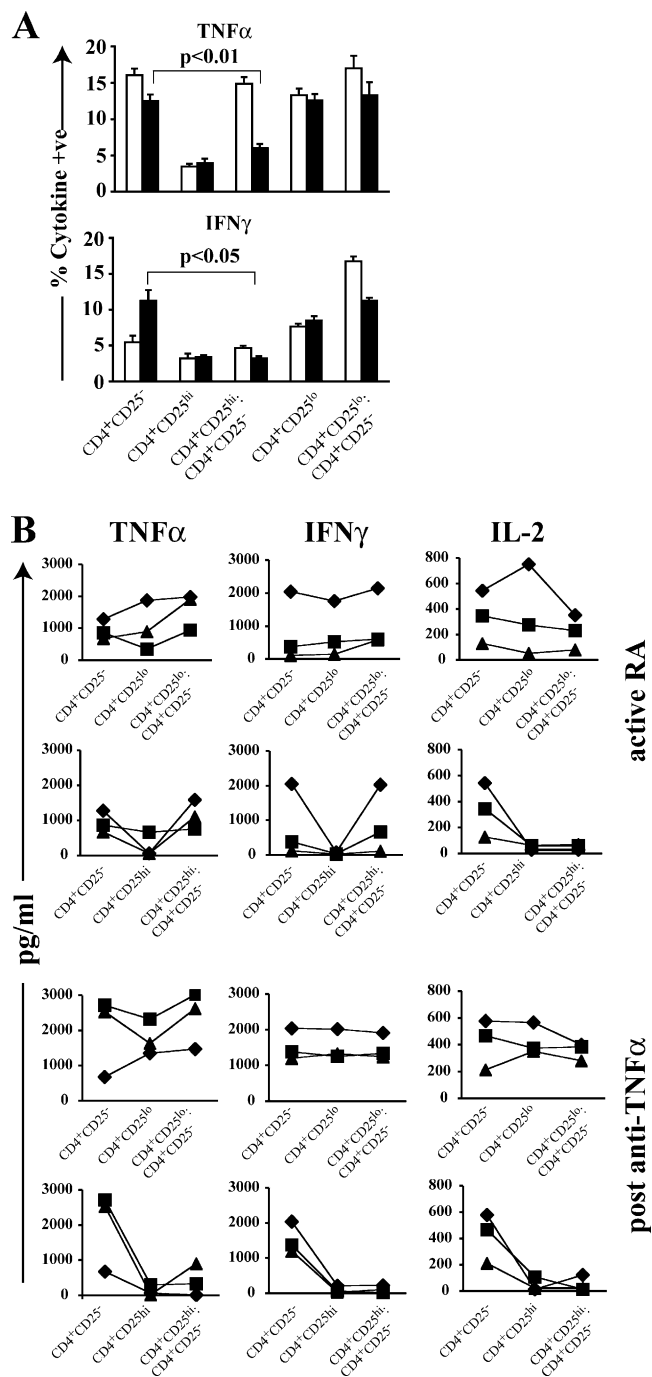


Figure 2. The regulatory defect resides within the $CD4^+CD25^{high}$ T cell population. (A) The $CD4^+CD25^-$, $CD4^+CD25^{low}$, $CD4^+CD25^{high}$ population were FACS[®]-sorted (MoFlo) from an active RA patient before treatment (white columns) and a responding RA patient (black columns) receiving anti-TNF α therapy using the indicated gates is depicted in Fig. S1. The $CD4^+CD25^{high}$ and the $CD4^+CD25^{low}$ T cells were cultured alone or with $CD4^+CD25^-$ responder T cells (5×10^4 cells/well) and stimulated with 5 μ g/ml of soluble anti-CD3/anti-CD28 for 48 h. 2 μ M monensin was added for the last 5 h of culture. Cells were stained to detect intracellular TNF α and IFN γ in a similar fashion to those in Fig. 1 B. Mean $\pm$ SEM of three patients before and after anti-TNF α therapy is shown. (B) Cytokines from the supernatants collected from the experiments from the three patients before and after anti-TNF α treatment (A) were measured by CBA and shown separately in each of the panels.

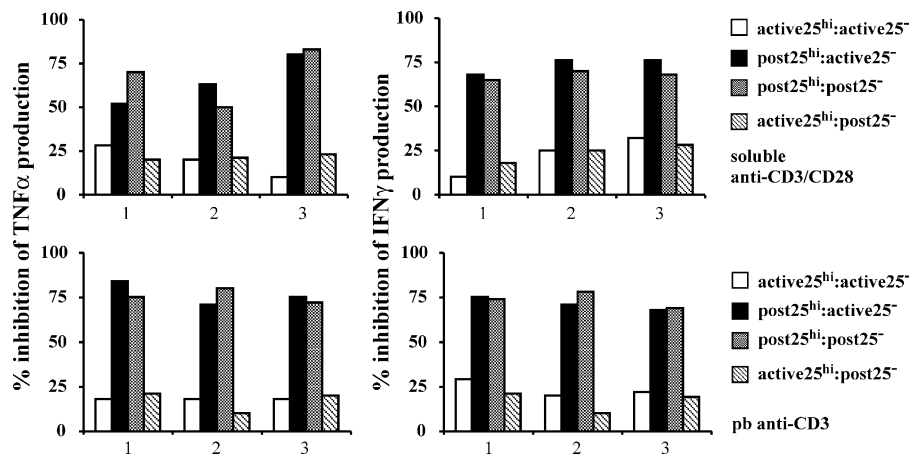


Figure 3. CD4⁺CD25^{high} T cells from patients with active RA fail to suppress cytokines produced by CD4⁺CD25⁻ T cells derived from the same patient either before or after anti-TNFα therapy. CD4⁺CD25^{high} and CD4⁺CD25⁻ T cells (5×10^4 cells/well) isolated from patients with active RA ($n = 3$) or from patients responding to anti-TNFα therapy ($n = 3$) were mixed and stimulated with 5 μg/ml of soluble anti-CD3/anti-CD28 (top) or with 1 mg/ml of plate-bound anti-CD3 (bottom). Supernatants were analyzed by CBA. The results indicate the percentage of inhibition of the cytokine production relative to the CD4⁺CD25⁻ T cells stimulated alone. One out of two experiments is shown.

these results demonstrate that regulatory T cells isolated from patients with active RA are “compromised” at least in terms of their ability to suppress proinflammatory cytokine production by responder T cells as well as from monocytes.

Next, we investigated the possibility that effector T cells are resistant to the suppressive effect exerted by the regulatory T cells in patients with RA. CD4⁺CD25⁻ or CD4⁺CD25^{high} T cells from patients before therapy were cultured with either CD4⁺CD25^{high} T cells or with CD4⁺CD25⁻ T cells isolated 3 mo after therapy. The results in Fig. 3 show that CD4⁺CD25^{high} T cells from active RA patients were unable to suppress the production of cytokines released by effector T cells isolated after anti-TNFα treatment. In contrast, 3 mo after anti-TNFα therapy, CD4⁺CD25^{high} T cells regained their ability to inhibit IFNγ and TNFα released by autologous effector T cells from active RA patients. The same conclusion could be drawn whether the cultures were stimulated with soluble anti-CD3/anti-CD28 (Fig. 3, top) or plate-bound anti-CD3 (Fig. 3, bottom). Therefore, our data demonstrate that CD4⁺CD25^{high} T cells are functionally defective in RA patients and the lack of suppression is not due to effector T cell resistance.

In the next experiment, we wished to establish whether the recovery in function mediated by anti-TNFα therapy is cell contact dependent and cytokine independent. In coculture experiments, freshly isolated CD4⁺CD25⁻ T cells were separated in transwell chambers from CD4⁺CD25^{high} T cells. Prevention of cell contact abolished their suppressive capacity (Fig. 4 A). Next, we investigated whether addition or neutralization of TNFα in vitro affects regulatory T cells. The results in Fig. 4 B demonstrate that the addition of TNFα to the coculture system did not alter the suppressive function of regulatory T cells isolated after anti-TNFα treatment. In vitro neutralization of TNFα (Infliximab) was not sufficient to restore the defect in CD4⁺CD25^{high} T cell-mediated suppression (unpublished data).

Depletion of CD4⁺CD25⁺ T Cells from PB Isolated from Anti-TNFα-treated Patients Results in an Increase in TNFα Levels and in an Inhibition of IL-10 Production. Next, we evaluated how CD4⁺CD25⁺ T cell depletion influences

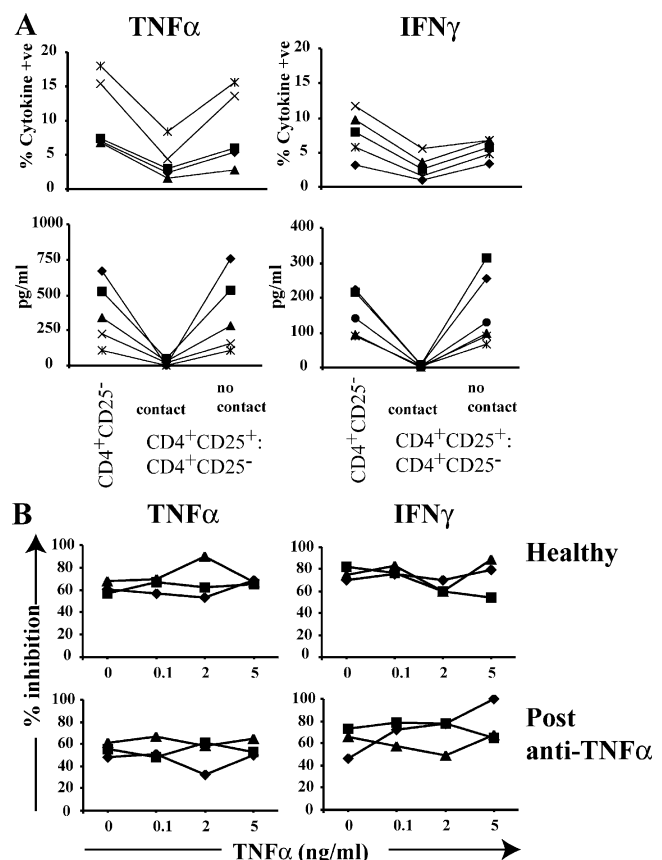


Figure 4. The inhibitory effect of CD4⁺CD25^{high} T cells requires cell contact and is not affected by the addition of TNFα in vitro. (A) CD4⁺CD25^{high} and responder T cells derived from patients responding to anti-TNFα therapy were stimulated with 5 mg/ml of soluble anti-CD3/anti-CD28 either with or without the use of a transwell membrane to prevent cell contact. (top) 2 μM monensin was added for the last 5 h of culture, and cells were stained for detection of intracellular cytokine production. (bottom) Cytokines from the supernatants collected from the same experiment were measured by CBA. (B) CD4⁺CD25^{high} and CD4⁺CD25⁻ T cells isolated from healthy as well as responding patients were cultured 1:1 ratio, upon anti-CD3/anti-CD28 stimulation, in the presence of different concentrations of TNFα. The percentage of suppression of cytokine production was calculated in each individual. One out of two experiments is shown.

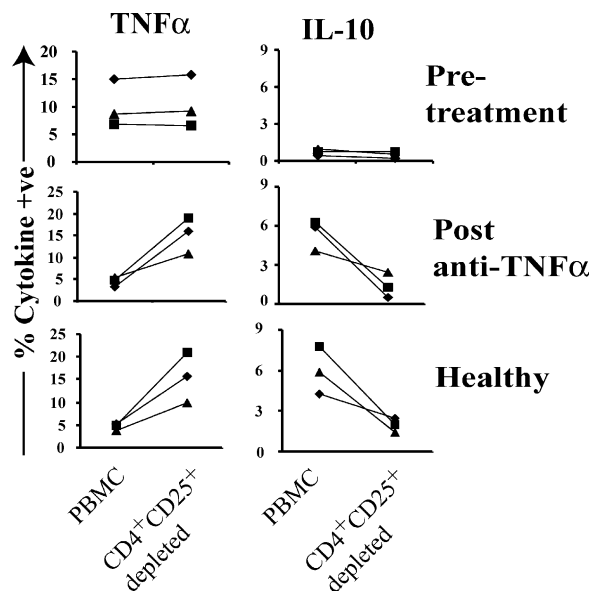


Figure 5. Effect of depletion of $CD4^+CD25^+$ T cells from PBMCs on $TNF\alpha$ and IL-10 production from the same patient before and after treatment. Total PBMCs or PBMCs depleted of $CD25^+$ cells (by MACS® sorting) were stimulated with 5 μ g/ml of soluble anti-CD3/anti-CD28 for 48 h. 2 μ M monensin was added for the last 5 h of culture. Staining of CD4 and CD25 surface expression was performed. Cells were permeabilized and stained with PE-conjugated anti- $TNF\alpha$ or anti-IL-10. Results from three patients before and after therapy and three healthy individuals are shown.

the cytokine profile of PB before and after anti- $TNF\alpha$ treatment. PB from patients with active RA synthesized high levels of $TNF\alpha$, but no IL-10 (Fig. 5). After treatment, a reduced amount of $TNF\alpha$ and an increased number of IL-10-secreting cells were detected in PB. After depletion of $CD4^+CD25^{high}$ T cells from PB derived from patients with active RA, there was no change in the percentage of cells producing $TNF\alpha$ or IL-10, whereas a fivefold increase in $TNF\alpha$ and a >10-fold reduction in IL-10 production occurred when regulatory T cells were depleted from anti- $TNF\alpha$ -treated RA patients. Analysis of PB from healthy individuals gave similar results to patients responding to anti- $TNF\alpha$ therapy. These results provide additional evidence that regulatory T cells from patients with active RA are ineffective in terms of suppressing $TNF\alpha$ release and that this function is restored after treatment.

$CD4^+CD25^+$ Regulatory T Cells from Active RA Patients Lack the Ability to Convey Suppressor Activity to Effector T Cells: Restoration after Anti- $TNF\alpha$ Treatment. The generation of newly formed regulatory T cells may be a further mechanism by which regulatory T cells could control autoreactive T cells and the consequent inflammation. It has been shown recently that regulatory T cells, isolated from healthy individuals, convey a suppressive phenotype to "conventional" activated T cells (19). To explore this possibility in patients with RA, we tested the capacity of regulatory T cells, isolated before and after treatment, to convey a suppressive phenotype to $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ T cells were isolated as previously men-

tioned and cultured 1:1 with the $CD4^+CD25^-$ T cell fraction in the presence of soluble anti-CD3/anti-CD28. After 3 d, $CD4^+CD25^+$ T cells were depleted, and the remaining negative fraction ($CD4^+CD25^-$) was cultured 1:1 with freshly isolated autologous $CD4^+CD25^-$ T cells for an additional 5 d. In agreement with results reported from Jonuleit et al. (19), the resulting $CD4^+CD25^-$ T cell fraction purified from healthy or anti- $TNF\alpha$ -treated patients suppressed the proliferation of freshly isolated autologous T cells (Fig. 6). In contrast, similarly prepared $CD4^+CD25^-$ T cells from patients with active RA showed no suppressive activity on freshly isolated T cells, suggesting that regulatory T cells from these patients are unable to imprint a "suppressive" phenotype onto the $CD4^+CD25^-$ T cells. As an additional control, we also depleted $CD4^+CD25^+$ T cells (which should represent the newly activated T cells) from the $CD4^+CD25^-$ T cells cultured alone, and replated with freshly isolated $CD4^+CD25^-$ T cells. No suppressive phenotype was acquired under these experimental conditions (unpublished data), confirming that this phenomenon is regulatory T cell dependent. These results demonstrate that regulatory T cells isolated from patients with active RA are unable to inhibit the production of proinflammatory cytokines and to induce the differentiation of additional populations of suppressor cells.

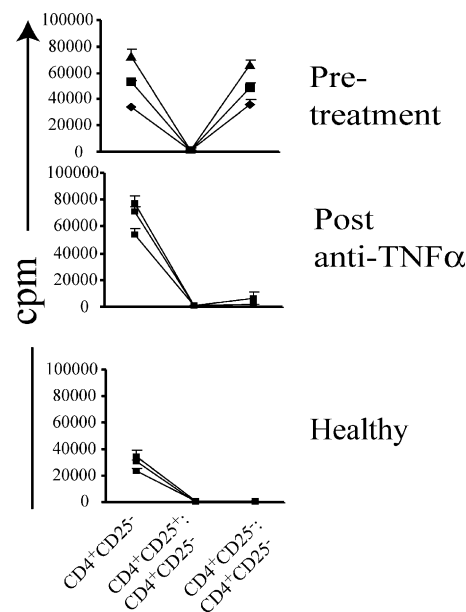


Figure 6. Only $CD4^+CD25^+$ regulatory T cells derived from patients treated with anti- $TNF\alpha$, but not from patients with active RA, convey a suppressive phenotype to effector T cells. MACS®-sorted $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were cultured either alone or mixed at a 1:1 ratio (10^5 cells/well) and stimulated with 1 μ g/ml of soluble anti-CD3 and 2 μ g/ml anti-CD28. After 5 d of culture, $CD4^+CD25^+$ T cells were depleted and the remaining $CD4^+CD25^-$ T cells were replated 1:1 with freshly isolated autologous $CD4^+CD25^-$ T cells in the presence of anti-CD3 and anti-CD28 for an additional 3 d (third column). ^3H Tdr was added for the final 8 h of culture (expressed as mean $\pm$ SEM of triplicate cultures). Results from three patients before and after therapy and three healthy individuals are shown.

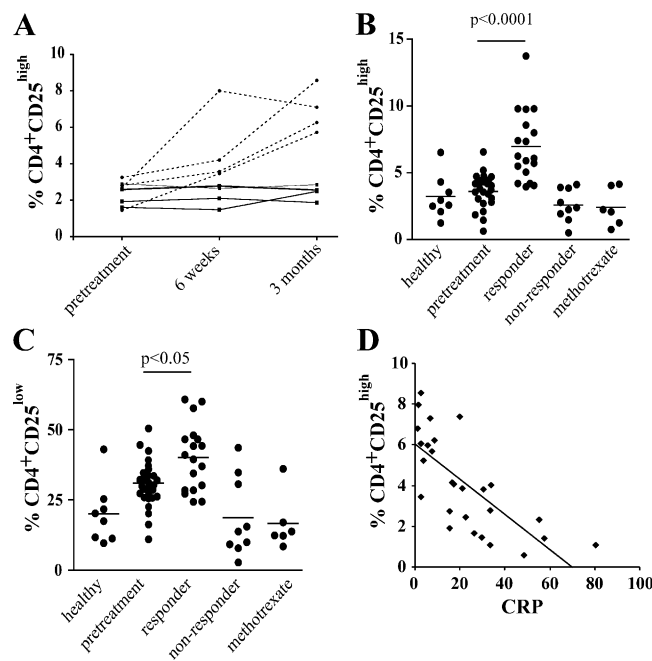


Figure 7. Increase in CD4⁺CD25^{high} T cells in the PB of RA patients responding to anti-TNF α therapy. (A) The percentage of CD4⁺ T cells expressing CD25^{high} was followed in four anti-TNF α responder (dashed line) and four nonresponder (solid line) patients with RA before and after treatment. (B) Analysis of larger group of patients treated with anti-TNF α ($n = 27$), methotrexate alone ($n = 6$), and healthy controls ($n = 8$). CD4⁺CD25^{high} and (C) CD4⁺CD25^{low} T cells (as defined in Fig. S1 A). (D) Scatter plot showing a significant correlation between the C reactive protein and the percentage of CD4⁺CD25^{high} T cells before and after treatment ($P < 0.001$, $r^2 = 0.5278$).

The Frequency of CD4⁺CD25^{high} T Cells Is Higher in Anti-TNF α -responding Patients Compared with the Same Patients before Treatment. Finally, we monitored the numbers of regulatory T cells in patients with RA, before and after treatment (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20040165/DC1>). The expression of CD25^{high/low} on CD4⁺ T cells was initially examined in four responding and four nonresponding patients during the first 3 mo of treatment using FACS[®] analysis. Although the percentage of CD4⁺CD25^{high} T cells increased over time in those patients who responded to anti-TNF α therapy, there was no change in the percentage of CD4⁺CD25^{high} T cells in the four nonresponding patients (Fig. 7 A). The percentage of this population continued to rise in three out of the four responding patients 6 wk after the first infusion of Infliximab and remained stable after 3 mo of treatment (unpublished data). The subsequent analysis in a larger cohort of patients, including those responding to methotrexate therapy, revealed that the percentage of both CD4⁺CD25^{high/low} T cells was significantly increased in anti-TNF α -responding patients compared with the levels measured in those with active RA, methotrexate-treated patients, and healthy controls (Fig. 7, B and C). Active RA patients also showed an increased percentage of CD4⁺CD25^{low} T cells, which likely reflects increased activation of CD4⁺ T cells, and does not represent any changes

in the regulatory T cell population, in accordance with our (Fig. 2) and previously published data indicating that the regulatory CD4⁺ T subset is largely found within the CD25^{high} subset (10). The methotrexate-treated individuals were matched to the responding anti-TNF α -treated patients with respect to their response to treatment. Consistent with previous results, no reduction in numbers of regulatory T cells was measured in patients with active RA compared with healthy individuals (17), suggesting that numerical deficiency is unlikely to be responsible for any break in immune tolerance in RA, as has been suggested in some mouse models of autoimmune disease (3, 4), and in patients with type 1 diabetes mellitus (14). No differences in CD4⁺CD25^{high/low} expression were detected in patients with RA who failed to respond to anti-TNF α therapy compared with healthy controls. A significant correlation between the CRP, a measure of rheumatoid disease activity, and the percentage of CD4⁺CD25^{high} T cells (but not CD4⁺CD25^{low}) confirmed the relationship between a response to anti-TNF α therapy and an expansion in this cell population (Fig. 7 D). In agreement with previous papers, there was no significant change in the absolute numbers of CD4⁺ T cells in PBMCs before treatment and 3 mo after anti-TNF α treatment, indicating that the changes in percentages observed in CD4⁺CD25⁺ T cells reflected alterations in the absolute numbers of cells (20).

Discussion

The data presented here provide new insight into the biology of regulatory T cells within the context of a human autoimmune disease. CD4⁺CD25^{high} T cells isolated from patients with active RA, although still anergic, show compromised function as demonstrated by their inability to regulate proinflammatory cytokines released by effector T cells and monocytes. After Infliximab treatment, regulatory T cell-mediated suppression was restored to the level found in healthy individuals, whereas only a partial restoration was seen in regulatory T cells isolated from patients responding to methotrexate. Although it is well documented that Infliximab blocks both soluble and transmembrane TNF α , resulting in a strong inhibition of other proinflammatory cytokines, there is no unanimity about the effects of methotrexate on cytokine production in RA (21, 22). If proinflammatory cytokines are not efficiently suppressed in methotrexate-treated patients, this could have a deleterious effect on the function of regulatory T cells, thus explaining the different level of regulatory T cell-mediated inhibition in patients treated with these two therapies.

It is unclear why regulatory T cells isolated from RA patients are unable to suppress lymphocyte and monocyte cytokine production. One possibility is that the presence of TNF α as well as other proinflammatory cytokines may hinder the ability of regulatory T cells to prevent autoimmune disease. In a recent paper that examined the role of this cytokine with respect to regulatory T cells, TNF α impaired the ability of regulatory T cells to suppress disease in

the NOD mouse model of diabetes (4). These authors suggested that endogenous levels of TNF α might act centrally in the thymus to mediate these effects on regulatory T cells. Pertinent to their hypothesis is the recent observation that CD4⁺CD25⁺ T cells derived from the thymus of healthy donors have an increased expression of TNFRII compared with CD4⁺CD25⁻ T cells (12), which might result in an increased susceptibility of regulatory T cells to the actions of TNF α . However, our in vitro data do not lend support to a direct effect of TNF α on regulatory T cells because, after exposure to a gradient of TNF α concentration alone, regulatory T cells were still viable and functionally active. These results indicate that more complex mechanisms are likely to operate in vivo. That regulatory T cells isolated from patients affected by chronic inflammatory disorders show altered function is not only peculiar to RA. Indeed, it has been reported recently that regulatory T cells isolated from the PB of patients with multiple sclerosis are functionally defective in terms of their ability to suppress proliferation and cytokines production by activated T cells (23). This finding, together with our data, might shed some light on the role that regulatory T cells play in controlling the maintenance of peripheral tolerance and in the prevention of human autoimmunity.

It has been speculated previously that the spreading of suppression from CD4⁺CD25⁺ T cells to responder T cells is an important mechanism by which regulatory T cells exert their effect in the maintenance of peripheral tolerance (19). This is an attractive explanation for how such small numbers of cells could prevent autoimmune disease. Our in vitro data suggest that, unlike regulatory T cells from healthy individuals, those derived from active RA patients cannot convey a suppressive phenotype to activated T cells. To our knowledge, this is the first time that this phenomenon has been shown to be impaired in an autoimmune disease. This defect could have a significant impact on the regulation of RA pathology because it is likely that the generation of newly formed (and fully functional) suppressor T cells is critical to control autoimmunity.

In searching for the mechanism by which these regulatory T cells act, we found that the suppression of cytokine production by regulatory T cells after treatment was contact dependent, and soluble factor independent as neutralization of IL-10 and TGF β did not alter their suppressive function (unpublished data). However, depletion of CD4⁺CD25⁺ T cells from treated PB led to a reduction in the number of IL-10-secreting cells. This suggests that the production of IL-10 may be dependent on the presence of regulatory T cells, but that its release is by another population such as a subset of suppressor cells that do not express CD25. It has been suggested that CD4⁺CD25⁺ T cells may actively promote the differentiation of Tr1 cells that mediate their suppression through production of IL-10 (24). As IL-10 is widely known for its antiinflammatory properties, it is tantalizing to hypothesize that the increase in this cytokine, previously reported in anti-TNF α -treated patients (20, 25), is orchestrated by regulatory T cells whose

function has been restored. Interestingly, we were unable to detect IL-10 in any of the T cell populations analyzed, suggesting that the production of IL-10 depends on the interaction of a variety of cell types (and/or their soluble products) present in whole PBMCs.

The other major finding described in our paper is the increased regulatory T cell number in RA patients treated with anti-TNF α , which significantly correlated with a reduction in CRP value. It is tempting to speculate that the increase in regulatory T cells in responding patients is important in the amelioration of disease and may help to differentiate between responder and nonresponder patients because the changes occur within 6 wk of initiation of therapy. It is conceivable that TNF α has a direct effect on regulatory T cell viability, such as the induction of apoptosis (26), which would explain the increased number of regulatory T cells after TNF α neutralization. As aforementioned, our in vitro data do not support this hypothesis. Moreover, we found no reduction in regulatory T cell numbers in patients with active RA compared with healthy donors, suggesting that neither apoptosis, nor any other direct effect on regulatory T cell numbers, are the primary mechanisms by which TNF α is acting. The lack of difference in CD25^{high} regulatory T cell numbers found in active patients, compared with healthy individuals, needs more careful study, as the CD25^{high} regulatory T cells derived from active RA could be "contaminated" by recently activated CD4⁺ T cells, which would mask the total number of regulatory T cells. Indeed, human CD4⁺CD25⁺ T cells are not a homogenous population and contain nonsuppressive fractions (10, 27). Of equal importance to the issues already raised is the possibility that an increase in regulatory T cells may have deleterious consequences, in particular an increased susceptibility to infections, which is a major issue in patients receiving anti-TNF α therapy (28).

Further studies are required to elucidate the mechanisms that underlie these observations in patients with RA, and to relate the clinical and immunological responses in patients receiving anti-TNF α therapy. Restoration of the function of regulatory T cells or transfer of fully competent regulatory T cells could be a useful therapeutic tool in the treatment of RA (29).

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