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Harnessing immune regulation for treatment of human diseases : CD4+CD25+ regulatory T cells & antibody glycosylation

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CHAPTER 3

Suppressor activity among $CD4^+CD25^{++}$ T cells is discriminated by membrane-bound TNFα

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ABSTRACT

Objective. Previous reports have shown that the suppressive capacity of $CD4^+CD25^{++}$ T cells is compromised and reversed by anti-TNFα therapy in patients with rheumatoid arthritis (RA). Given the lack of specific cell surface markers for human T regulatory (Treg) cells, this study aimed to define surface makers for identifying and enriching Treg cells with enhanced regulatory ability within the $CD4^+CD25^{++}$ T cell compartment and to provide additional understanding of the effects of anti-TNFα antibodies in humans.

Methods. The expression of membrane-bound TNFα (mTNFα) in human peripheral blood $CD4^+$ T cells was analyzed by flow cytometry in healthy individuals and RA patients before/after anti-TNFα treatment. mTNFα positive and negative $CD4^+CD25^{++}$ T cells were purified by FACS-sorting and their suppressive capacity were assessed *in vitro* by a standard suppression assay.

Results. A substantial number of $CD4^+CD25^{++}$ T cells expressed mTNFα. $mTNF\alpha^+CD4^+CD25^{++}$ T cells displayed reduced anti-inflammatory cytokine production and less potent suppressor capacity, since 4 times more cells were required to achieve 50% inhibition compared with their $mTNF\alpha^-$ counterparts. Treatment of RA patients with TNFα-specific antibodies led to a reduction in the number of $mTNF\alpha^+CD4^+CD25^{++}$ T cells from peripheral blood.

Conclusion. Our data indicate that the absence of mTNFα on $CD4^+CD25^{++}$ T cells can be used to characterize and enrich for Treg cells with maximal suppressor potency. Enrichment of $mTNF\alpha^-CD4^+CD25^+$ cells in the $CD4^+CD25^{++}$ T cell compartment may contribute to restoring the compromised suppressive ability of $CD4^+CD25^{++}$ T cell populations in RA patients after anti-TNFα treatment.

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INTRODUCTION

$CD4^+CD25^+$ Treg cells are generated in the thymus and periphery and represent a crucial regulator of peripheral self-tolerance (1, 2). They are hyporesponsive to TCR stimulation *in vitro* (3, 4), however, once activated, they can suppress the proliferation and cytokine production of effector T cells, antibody production of B cells, and the function of monocytes/macrophages (3-6). Hence, $CD4^+CD25^+$ Treg cells have been successfully used to prevent/treat immunologic diseases in a variety of animal models, such as collagen-induced arthritis (CIA) (7-9).

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by chronic synovial inflammation resulting in cartilage and bone damage, eventually leading to joint destruction. Several different cell types and their mediators are thought to be involved in this tissue-destructive inflammation, including T cells, B cells and pro-inflammatory cytokines such as TNFα. The chronic inflammatory process suggests that immune regulation in RA patients is disturbed. Indeed, numerous reports have outlined disturbances in $CD4^+CD25^+$ T cell functions in patients with RA as well as other autoimmune diseases (10-15), suggesting an imbalance between Treg and effector T cell activities. However, functional $CD4^+CD25^+$ Treg cells have also been reported in RA patients (16, 17), which could be a consequence of the complex phenotype of these cells in humans and the lack of specific cell surface markers to define and isolate Treg cells.

Although CD25 appears to identify a relatively homogeneous population of Treg cells in naive mice, the presence of significant amounts of activated conventional $CD25^+$ T cells in humans makes it difficult to distinguish "genuine" Treg cells from activated conventional T cells in the $CD4^+CD25^+$ T cell compartment. Indeed, several studies have suggested that only the $CD4^+$ T cell subset expressing the highest levels of CD25 (termed $CD25^{high}$ or $CD25^{++}$) is mostly $Foxp3^+$ and has *in vitro* suppressive activity (18). Nonetheless, activated "conventional" T cells may still be present in the $CD4^+CD25^{++}$ T cell subset, because Foxp3, the most commonly used marker for Treg cells (19), is also transiently expressed in activated nonsuppressive T cells (20-22). For this reason, the $CD4^+CD25^{++}$ T cell population that homogenously expresses Foxp3 might not consist entirely of Treg cells but could also contain conventional activated $CD4^+CD25^{++}$ T cells, especially in situations in which chronic T cell activation is thought to occur. The possible "contamination" of Treg cells with "conventional" $CD4^+CD25^{++}$ T cells might also contribute to the compromised suppressive activity of $CD4^+CD25^{++}$ T cell populations in autoimmune diseases such as RA (10-15), in which chronic T cell activation has been implicated. Likewise, other surface markers, such as CTLA-4 and GITR, which have been reported to be expressed on Treg cells (23, 24), do not distinguish Treg cells from activated effector cells in humans. Therefore, they can not be used as such to identify Treg cells, especially not in conditions with chronic T cell activation. Hence, further characterization and therapeutic use of Treg cells would be greatly facilitated by the identification of surface markers that could be used to define and isolate Treg cells with high suppressive activity.

TNFα is a pleiotropic cytokine that is bioactive both as a transmembrane protein as well as a homotrimeric secreted molecule (25). Membrane-bound TNFα (mTNFα) is expressed on normal human peripheral blood T cells after activation (26). As part of a range of proteins that are expressed on the cell surface after T cell activation, mTNFα on $CD4^+$ T cells can modulate the activation of macrophages and costimulate B cells, thereby enhancing their antibody production (27-29). Because it is conceivable that these latter features are mediated by conventional effector T cells rather than by Treg cells, and given that T cells also upregulate CD25 upon activation, we reasoned that mTNFα-expressing

$CD4^+CD25^{++}$ T cells are enriched for activated conventional T cells rather than for naturally occurring Treg cells. In that case, mTNFα positive $CD4^+CD25^{++}$ T cells would differ in phenotype and/or functional activity when compared with their $mTNF\alpha^-$ counterparts.

In the present study, we show that the expression of mTNFα on $CD4^+CD25^{++}$ T cells is correlated with disease activity in RA patients. Although mTNFα positive and negative $CD4^+CD25^{++}$ T cells express comparable levels of Foxp3 and are both hyporesponsive to TCR stimulation, $mTNF\alpha^-CD4^+CD25^{++}$ T cells produce more anti-inflammatory cytokine IL-10 and suppress the proliferation and cytokine production of responder T cells in a more profound manner compared with their $mTNF\alpha^+$ counterparts. Furthermore, treatment with anti-TNFα antibodies results in a reduced frequency of $mTNF\alpha^+$ T cells in the $CD4^+CD25^{++}$ T cell compartment in RA patients, suggesting that selective depletion of the less suppressive $mTNF\alpha^+CD4^+CD25^{++}$ T cells by anti-TNFα antibodies contributes to the restoration of Treg activity in RA patients that has been reported previously (10-12, 30).

MATERIALS AND METHODS

Samples. Fresh peripheral blood was obtained from healthy adult donors with no history of autoimmune diseases. Fresh peripheral blood from RA patients was obtained from patients visiting the outpatient clinic of the Leiden University Medical Center. All RA patients fulfilled the 1987 revised classification criteria of the American College of Rheumatology (31). The characteristics of these RA patients are summarized in Table 1. Buffy coats were obtained from the Sanquin Bloodbank. Informed consent was provided in accordance with procedures approved by the local human ethics committee.

Table 1. Characteristics of and disease parameters for the RA patients*

Characteristics	Total
Age, years	55.2 ± 14.5
RA duration, years	5.55 ± 2.79
Disease activity score 44 (DAS44)†	1.60 ± 0.88
ESR, mm/hour†	17.6 ± 15.6
No. of women/no. of men	22/18
No. RF positive/no. RF negative‡	22/5
No. anti-CCP positive/no. anti-CCP negative‡	18/9

* Except where indicated otherwise, values are the mean ± SEM.
† Data on the Disease Activity Score (**DAS**) and erythrocyte sedimentation rate (**ESR**) were available for 23 patients when the mTNFα expression was analyzed.
‡ Data on the rheumatoid factor (**RF**) and anti-cyclic citrullinated peptide (**anti-CCP**) status were available for 27 patients when the mTNFα expression was analyzed.

Antibodies. FITC-conjugated anti-CD25 (2A3), PE-conjugated anti-CD25 (M-A251), anti-CD127 (hIL-7R-M21), anti-CD45RO (UCHL1), APC-conjugated anti-CD4 (SK3), anti-CD25 (M-A251) and Percp-Cy5.5-conjugated anti-CD4 (SK3) were all from BD Biosciences. PE/FITC-coupled anti-Foxp3 (PCH101; eBiosciences) was used as indicated. Anti-TNFα mAb (infliximab) was labeled with FITC (Calbiochem) and used to detect the expression of mTNFα on cells.

Cell isolation. PBMCs were isolated from peripheral blood or buffy coats by centrifugation over Ficoll-Hypaque gradients. $CD4^+$ T cells were enriched from PBMC by negative selection with the $CD4^+$ T cell isolation kit (Miltenyi Biotec). $CD4^+$ T cells were then stained with anti-$CD4^{APC}$, anti-$CD25^{PE}$ and anti-$TNF\alpha^{FITC}$ for 30 minutes at 4 °C. After washing, $CD4^+CD25^-$ responder T cells, $mTNF\alpha^+$ and $mTNF\alpha^-$ cells within the $CD4^+CD25^{++}$ T cell population were isolated by sorting with a Flow Sorter Aria machine (BD Biosciences). The sorted cells were gated first on small lymphocytes by forward and side scatter, then on CD4 and $CD25^{++}$ (top 2-5% of $CD4^+$ T cells) expression, and then on mTNFα expression. CD4-depleted PBMC, irradiated with 4500 rads were used as feeders.

Flow cytometric analysis. Single-cell suspensions were prepared, and surface molecules were stained for 30 minutes at 4 °C with optimal dilutions of each antibody. After fixation and permeabilization, cells were incubated with anti-Foxp3 antibody. Expression of cell-surface and intracellular markers was assessed using flow cytometry (FACSCalibur; Becton Dickinson, California) after gating on live cells determined by scatter characteristics. Data were analyzed by CellQuest Pro software (Becton Dickinson).

Proliferation and suppression of T cells. Cells were plated at 1 x 10^4 cells/well in 96-well plates with 1 μg/ml phytohemagglutinin (PHA) and feeders (5 x 10^4 cells/well) in the presence or absence of 50 U/ml IL-2. Cells were pulsed with ^{3}H-thymidine (0.5 μCi/well) on day 4, and proliferation was assessed 18 hours later using a liquid scintillation counter. To test for suppressive capacity, $CD4^+CD25^-$ responder T cells were stimulated as described above without the addition of exogenous IL-2. Autologous $mTNF\alpha^+CD4^+CD25^{++}$ or $mTNF\alpha^-CD4^+CD25^{++}$ T cells were added, and suppression was assessed by determining ^{3}H-thymidine incorporation as well as by measuring the amounts of TNFα and IFNγ in culture supernatants.

Cytokine detection. To determine the concentration of cytokines, BD CBA Flex Sets (for IL-10 and TNFα) (BD Biosciences) or a capture ELISA (for IFNγ) was used to analyze culture supernatants after 96 hours, following the protocol provided by the manufacturer. The beads were analyzed on a LSRII machine using the BD FCAP Array software (BD Biosciences).

Statistical analysis. The Mann-Whitney U test was used to compare Treg-mediated suppression results and cellular frequencies in healthy controls and RA patients. Paired *t*-tests were used to compare measurements of RA patients before and after anti-TNFα treatment. Spearman's correlations were used to compare cellular frequencies with subject age. All statistical analyses were performed using GraphPad Prism 4.00 software (GraphPad, San Diego, CA). *P* values less than 0.05 were considered significant.

RESULTS

Preferential expression of mTNFα on CD4$^+$CD25^{++} T cells in vivo

Recent findings have indicated that the suppressive function of CD4$^+$CD25^{++} T cells is compromised in patients with active RA (10, 11). However, the suppressive capability of the CD4$^+$CD25^{++} T cell population is reversed after anti-TNFα therapy (10-12, 30). Given the prominent role of TNFα in inflammation and in inflammatory autoimmune diseases such as RA (32-34), and given the effector functions of mTNFα on activated CD4$^+$ T cells (i.e., macrophage activation and increment of antibody production) (27-29), it is conceivable that the mTNFα-expressing CD4$^+$CD25^{++} T cell population is enriched for "conventional" activated T cells.

We therefore hypothesized that this T cell population possesses inferior suppressive abilities compared with the non-mTNFα-expressing CD4$^+$CD25^{++} T cell population. Furthermore, we speculated that mTNFα$^+$CD4$^+$CD25^{++} T cells are preferentially depleted following anti-TNFα therapy, which in turn could contribute, at least partially, to the reappearance of the suppressive function of isolated CD4$^+$CD25^{++} T cell populations from RA patients after anti-TNFα therapy (10-12, 30). Hence, we wished to examine the phenotype and function of mTNFα-expressing cells in more detail to determine whether these characteristics could be used to distinguish T cells with different suppressive potency within the CD4$^+$CD25^{++} T cell compartment. To this end, we first characterized mTNFα expression on freshly isolated human CD4$^+$ T cells.

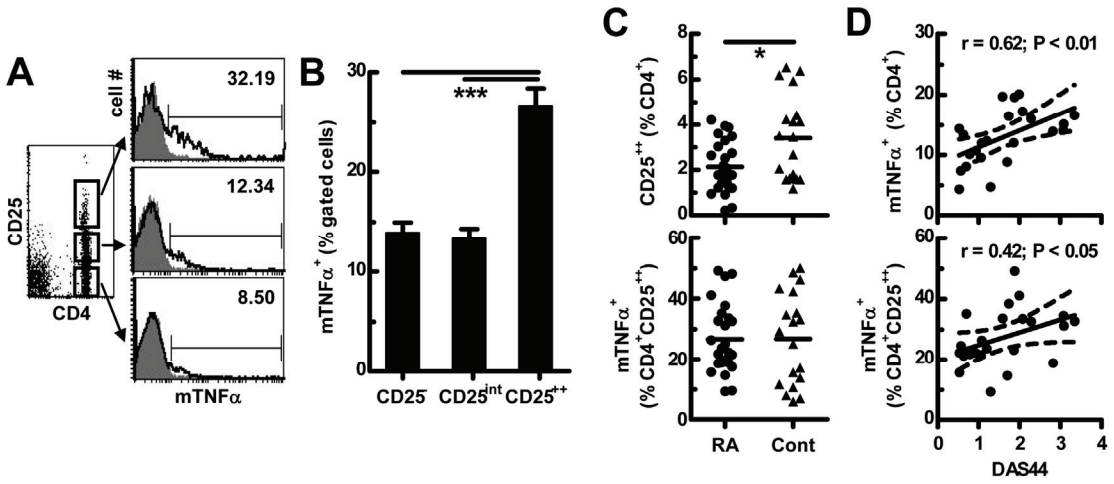

Figure 1. Correlation of mTNFα expression with disease activity in patients with RA. PBMCs were isolated from RA patients and stained with anti-CD4, anti-CD25, and anti-TNFα (infliximab). CD25 and mTNFα expression was monitored by flow cytometry. All patients analyzed were naive for TNFα-antagonists. **(A)** Representative histogram graph (*right panel*) showing mTNFα staining (black line) and isotype staining (shaded area) within each cell population. The gates for CD4$^+$CD25$^-$ (*bottom box*), CD4$^+$CD25^{int} (*middle box*) and CD4$^+$CD25^{++} (*top box*) T cells are demonstrated in the *left panel*. The upper-right numbers indicate the percentage of positive cells within each gate. **(B)** Bar graph of the percentage of mTNFα-expressing cells in different T cell populations. Results are expressed as means ± SEM from 40 different individuals. *** = $P < 0.001$. **(C)** Comparison of CD4$^+$CD25^{++} (*upper*) or mTNFα-expressing CD4$^+$CD25^{++} T cells (*lower*) between 26 RA patients (mean ± SEM age 46.7 ± 10.9 years) and 19 age-matched healthy controls (Cont) (mean ± SEM age 44.3 ± 13.8 years). Horizontal lines indicate median values for each group. * = $P < 0.05$. **(D)** Frequency of mTNFα-expressing cells within CD4$^+$ (*upper*) and CD4$^+$CD25^{++} (*lower*) T cell compartments, plotted against the Disease Activity Score (DAS) for 23 patients. Linear regressions (solid line) with 95% confidence intervals (dashed line) are shown.

PBMCs from RA patients were purified, and mTNFα expression was detected by flow cytometry. Similar proportions of $CD4^+CD25^-$ (mean 13.9%, [range 3.43-30.8]) and $CD4^+CD25^{int}$ (mean 13.3%, [range, 3.68-28.1]) T cells stained positive for mTNFα. However, within the $CD4^+CD25^{++}$ T cell compartment, a significantly higher percentage of $mTNF\alpha^+$ T cells (mean 26.6%, [range, 7.49-49.2]) was observed (Figure 1A & B). Similar results were obtained with cells isolated from healthy individuals (n=19, data not shown). Furthermore, a consistent increase in the percentage of $mTNF\alpha^+$ T cells was observed within the $Foxp3^+CD4^+$ T cell population compared with their $Foxp3^-CD4^+$ counterparts (data not shown). These results could not be explained by binding of the antibody to TNFα captured by surface TNFRs, because most $TNFRII^+CD4^+CD25^{++}$ cells are $mTNF\alpha^-$, and, more important, in line with previous reports (35), similar mTNFα expression was observed after pre-incubation of the cells with high concentrations of soluble TNFα (10 and 50 ng/ml) (data not shown). Together, these data indicate that human $CD4^+CD25^{++}$ T cells express mTNFα.

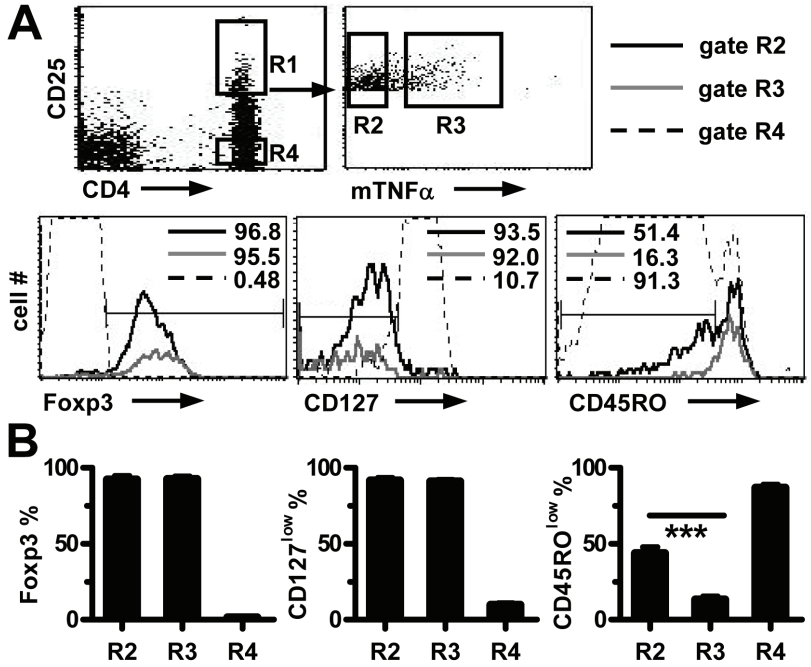

Figure 2. Expression of other markers on mTNFα positive and negative $CD4^+CD25^{++}$ T cells. PBMCs were stained with anti-CD4, anti-CD25 and anti-TNFα, together with anti-CD127, anti-CD45RO or anti-Foxp3 (intracellular). **(A)** Gating for histograms was performed on the basis of CD4, CD25 and mTNFα expression, as indicated in the *upper panel*. $mTNF\alpha^-CD4^+CD25^{++}$ (R2) and $mTNF\alpha^+CD4^+CD25^{++}$ (R3) T cell subsets were gated by first restricting to $CD4^+CD25^{++}$ T cells (R1). Percentages of $Foxp3^+$ (*bottom left*), $CD127^{-/low}$ (*bottom middle*) and $CD45RO^{-/low}$ (*bottom right*) cells within each gate (R2, black line; R3, gray line) are shown. $CD4^+CD25^-$ T cells (R4) were included as a control (dashed line). **(B)** Bar graph shows the percentages of $Foxp3^+$ (*left*), $CD127^{-/low}$ (*middle*) and $CD45RO^{-/low}$ (*right*) within each gate as shown in (**A**). Results are expressed as means ± SEM from 8 different individuals. *** = $P < 0.001$.

Correlation of mTNFα expression with disease activity in RA patients

We next wished to study whether the expression of mTNFα on $CD4^+CD25^{++}$ T cells differs between healthy controls and RA patients. To this end, we compared CD25 and mTNFα expression in 26 RA patients with that in age-matched healthy controls. Although the number of $CD4^+CD25^{++}$ T cells decreased significantly in these RA patients, there was

no significant difference in the frequency of mTNFα-expressing $CD4^+CD25^{++}$ T cells (Figure 1C).

The Disease Activity Score (DAS) (36) was available for 23 of the 40 RA patients studied at the same time that mTNFα expression was analyzed (Table 1). Given the role of TNFα in the pathogenesis of RA (32-34) and the expression and function of mTNFα on activated $CD4^+$ T cells (25-29), we wished to address the question of whether there is any association between the disease activity and the expression of mTNFα. As shown in Figure 1D, the analysis revealed that the percentages of $mTNF\alpha^+$ T cells within the $CD4^+$ ($r = 0.62$, $P < 0.01$) or $CD4^+CD25^{++}$ ($r = 0.42$, $P < 0.05$) T cell populations were correlated with disease activity in these RA patients. Given that mTNFα expression was also associated with erythrocyte sedimentation rate (ESR) in RA patients (data not shown), these data indicate that the expression of mTNFα is associated with the inflammatory immune response in RA patients. No association of its expression with disease duration was observed (data not shown).

Expression of other markers on mTNFα positive and negative $CD4^+CD25^{++}$ T cells

Given the association of mTNFα-expressing $CD4^+CD25^{++}$ T cells with disease activity (Figure 1D) and the possibly compromised suppressive activity of $CD4^+CD25^{++}$ T cells in patients with active RA, as previously reported (10, 11), we wished to characterize these mTNFα-expressing $CD4^+CD25^{++}$ T cells in more detail. Since Foxp3 is the most commonly used marker for human $CD4^+CD25^+$ Treg cells thus far (19), and because surface CD127 expression is inversely associated with the presence of Foxp3 and suppressive function of freshly isolated human $CD4^+$ T cells from healthy donors (37), we first determined how surface mTNFα expression correlates with Foxp3 and CD127 expression. Therefore, PBMCs were stained for CD4, CD25 and mTNFα, together with either Foxp3 or CD127.

As shown in Figure 2, no significant difference in intracellular Foxp3 expression was observed between mTNFα positive and negative $CD4^+CD25^{++}$ T cells, either in the percentage of positive cells or in the expression levels within each cell compartment. Likewise, a similar percentage of $CD127^{-/low}$ cells was observed within the $mTNF\alpha^-CD4^+CD25^{++}$ and $mTNF\alpha^+CD4^+CD25^{++}$ T cell subsets. Because the expression of CD45RO, the memory marker for human T cells, has been used to discriminate different $CD4^+CD25^+$ Treg populations (38), we analyzed the expression of this marker on mTNFα positive and negative $CD4^+CD25^{++}$ T cells as well. Almost all $mTNF\alpha^+CD4^+CD25^{++}$ T cells displayed high expression of CD45RO, whereas a significant proportion of their $mTNF\alpha^-$ counterparts had lower levels of CD45RO expression (Figure 2). Similar results were obtained when mTNFα positive or negative $CD4^+CD25^+$ T cells were analyzed (data not shown). Together, these data indicate that mTNFα expression on $CD4^+CD25^{++}$ T cells correlates with levels of CD45RO expression, despite similar Foxp3 and CD127 expression.

Association of mTNFα with decreased production of anti-inflammatory cytokines in $CD4^+CD25^{++}$ T cells

Human $CD4^+CD25^{++}$ Treg cells are hyporesponsive and produce relatively high levels of the anti-inflammatory cytokine IL-10 but low levels of pro-inflammatory cytokines such as TNFα and IFNγ (39). To analyze whether mTNFα expression on $CD4^+CD25^{++}$ T cells discriminates cells with differential cytokine production profiles, we purified $mTNF\alpha^+CD4^+CD25^{++}$ and $mTNF\alpha^-CD4^+CD25^{++}$ T cells from buffy coats by FACS, and

we subsequently stimulated them with PHA and autologous feeders in the presence or absence of exogenous IL-2.

With respect to proliferation and the production of pro-inflammatory cytokines TNFα and IFNγ, both mTNFα positive and negative $CD4^+CD25^{++}$ T cells were hyporesponsive compared with autologous $CD4^+CD25^-$ responder T cells (Figure 3A & B). Nonetheless, when mTNFα positive and negative $CD4^+CD25^{++}$ cells were compared side by side, $mTNF\alpha^+CD4^+CD25^{++}$ T cells produced more TNFα and IFNγ (Figure 3B) and displayed a higher rate of proliferation (Figure 3A). In contrast, both $CD4^+CD25^{++}$ T cell populations produced more IL-10 upon stimulation compared with $CD4^+CD25^-$ T cells (Figure 3B). Intriguingly, however, $mTNF\alpha^-CD4^+CD25^{++}$ T cells produced twice the amount of IL-10 in comparison with their $mTNF\alpha^+$ counterparts after stimulation in the presence of IL-2 (Figure 3B). The mean ± SEM IL-10 level produced by $mTNF\alpha^-CD4^+CD25^{++}$ T cells was 121.99 ± 14.27 pg/ml compared with 51.64 ± 7.68 pg/ml produced by their $mTNF\alpha^+$ counterparts after 4 days stimulation in the presence of IL-2 (n=4, $P < 0.05$, data not shown).

These results indicate a difference in functional capacity between these two $CD4^+CD25^{++}$ T cell subsets, which is also illustrated by the ratio of IL-10 to TNFα or IFNγ (Figure 3C). Together, these data point to the possibility that $mTNF\alpha^-CD4^+CD25^{++}$ T cells display a greater suppressive potency compared with their mTNFα-expressing counterparts.

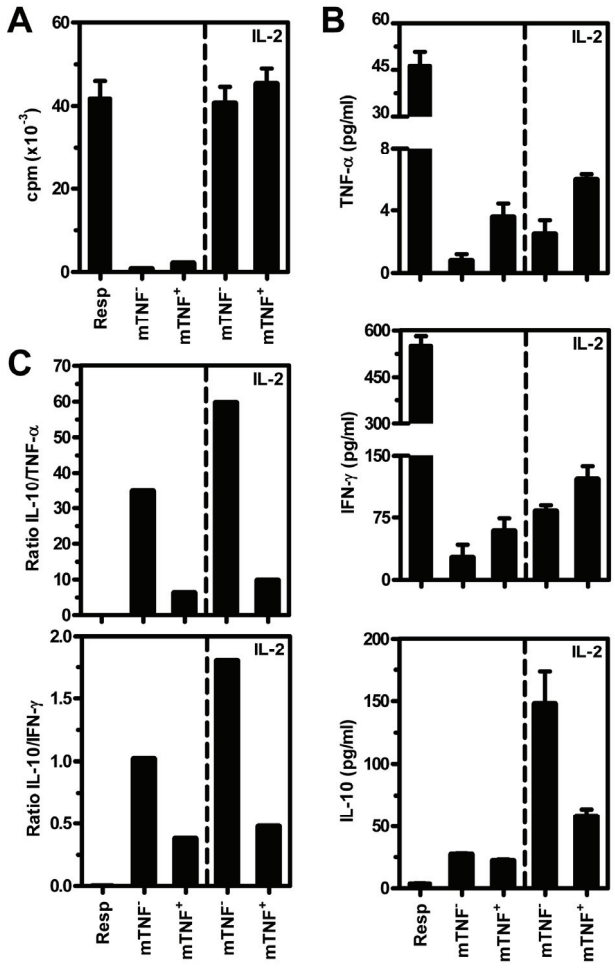

Figure 3. Association of mTNFα with decreased production of anti-inflammatory cytokines in $CD4^+CD25^{++}$ T cells. FACS-sorted $CD4^+CD25^-$ (Resp), $mTNF\alpha^-CD4^+CD25^{++}$ ($mTNF^-$) and $mTNF\alpha^+CD4^+CD25^{++}$ ($mTNF^+$) (1 x 10^4 cells/well) were activated with PHA (1 μg/ml) and feeders (5 x 10^4 cells/well) in the absence or presence of 50 U/ml exogenous IL-2. The sorting gates for $mTNF^-$ and $mTNF^+$ T cells are depicted in the upper panel of Figure 2. **(A)** Cell proliferation was determined by ^{3}H-thymidine uptake 5 days later. **(B & C)** Culture supernatants were collected after 96 h, and the amounts of TNFα (*upper*), IFNγ (*middle*) and IL-10 (*lower*) produced **(B)** or the ratios of IL-10/TNFα (*upper*) and IL-10/IFNγ (*lower*) **(C)** were determined. Results are expressed as mean ± SEM from 3 or 4 cultures and are representative of those from 4 independent experiments.

mTNFα on $CD4^+CD25^{++}$ T cells distinguishes a population with enhanced suppressor activity

To address whether differential mTNFα expression is indeed associated with differential suppressive activity, FACS-sorted $mTNF\alpha^+CD4^+CD25^{++}$ and $mTNF\alpha^-CD4^+CD25^{++}$ T cells were placed in a standard *in vitro* suppression assay using $CD4^+CD25^-$ as responder T cells. As shown in Figure 4, $mTNF\alpha^-CD4^+CD25^{++}$ T cells suppressed proliferation more robustly than did $mTNF\alpha^+CD4^+CD25^{++}$ T cells. Approximately 4-fold fewer $mTNF\alpha^-CD4^+CD25^{++}$ cells than $mTNF\alpha^+CD4^+CD25^{++}$ cells were required to achieve 50% suppression (Figure 4B).

To further confirm the differential suppressive capability between mTNFα positive and negative $CD4^+CD25^{++}$ cells, we next analyzed the amounts of TNFα and IFNγ in the coculture supernatants. Consistent with the results described above, the addition of $mTNF\alpha^-CD4^+CD25^{++}$ T cells at a 1:1 ratio resulted in a stronger reduction of TNFα and IFNγ production than the addition of $mTNF\alpha^+CD4^+CD25^{++}$ T cells (Figure 4C). This difference could not be attributed to the higher cytokine production by $mTNF\alpha^+CD4^+CD25^{++}$ T cells, since they secreted relatively small amounts of TNFα and IFNγ under these conditions (Figure 3B). Together, these observations indicate that $mTNF\alpha^-CD4^+CD25^{++}$ T cells possess an enhanced suppressive activity.

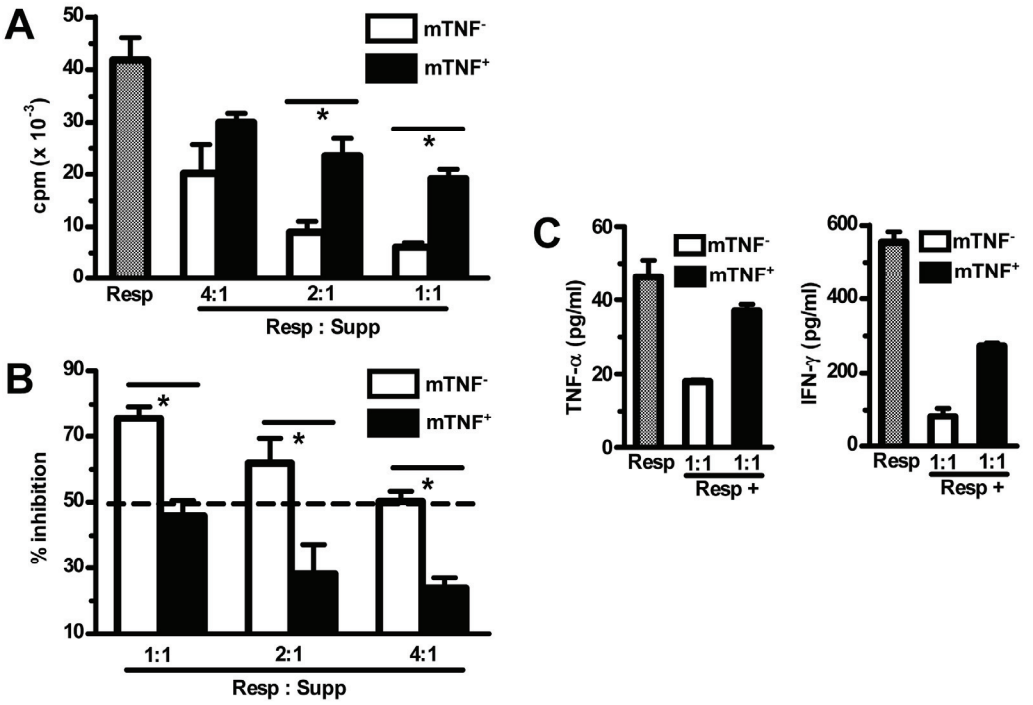

Figure 4. mTNFα on $CD4^+CD25^{++}$ T cells distinguishes a population with enhanced suppressor activity. FACS-sorted $CD4^+CD25^-$ responder T cells (Resp, 1 x 10^4 cells/well) were activated with PHA (1 μg/ml) and feeders (5 x 10^4 cells/well) in the absence or presence of $mTNF\alpha^-CD4^+CD25^{++}$ ($mTNF^-$) or $mTNF\alpha^+CD4^+CD25^{++}$ ($mTNF^+$) suppressor T cells (Supp) at a 4:1, 2:1 or 1:1 (Resp/Supp) ratio. In all experiments, $CD4^+CD25^-$ responder T cells were also added at a 1:1 ratio as a control (i.e., 2 x 10^4 responder T cells per well), which always yielded greater proliferation and cytokine production (data not shown). **(A)** Proliferation after 5 days was determined by ^{3}H-thymidine incorporation. **(B)** The average percent inhibition of proliferation is shown. The dashed line indicates 50% inhibition. **(C)** Culture supernatants were collected after 96 h and the amounts of TNFα and IFNγ were analyzed in cultures of responders only (Resp) or responders plus suppressors (Resp +) at a 1:1 ratio. Results are expressed as mean ± SEM from 3 or 4 cultures and represent **(A & C)** or summarize **(B)** 4 independent experiments with different individuals. *P < 0.05.

Decreased numbers of mTNFα$^+$ T cells in RA patients after anti-TNFα treatment

Previous studies have shown that anti-TNFα therapy could reverse the compromised function of isolated CD4$^+$CD25^{++} T cells in RA patients (10-12, 30). Given our observations that mTNFα$^+$CD4$^+$CD25^{++} T cells bear a less potent suppressor activity (Figure 4) and are associated with disease activity in RA patients (Figure 1D), we wished to know whether the expression of mTNFα on T cells was decreased in RA patients after TNFα-blocking therapy (adalimumab, n=7).

All these patients responded well to anti-TNFα treatment, as evidenced by a reduction in the mean ± SEM DAS in 28 joints (40), from 7.06 ± 0.56 to 3.89 ± 0.66 three months after treatment ($P < 0.0001$, data not shown). There was no difference in the absolute number and the frequency of CD3$^+$, CD4$^+$ T cells and CD4$^+$CD25^{++} T cells in peripheral blood (Figure 5A&B and data not shown) before and 3 months after adalimumab treatment. However, a significantly decreased expression of mTNFα on CD4$^+$CD25^{++} T cells (Figure 5A&B) as well as on total CD3$^+$ or CD4$^+$ T cells (data not shown) was observed in all patients. This could not be explained by the presence of adalimumab on mTNFα$^+$ T cells *in vivo* that would influence the detection of mTNFα expression *ex vivo* by infliximab, since the same expression levels of mTNFα were observed on cells regardless of whether they had been preincubated with adalimumab (Figure 5C).

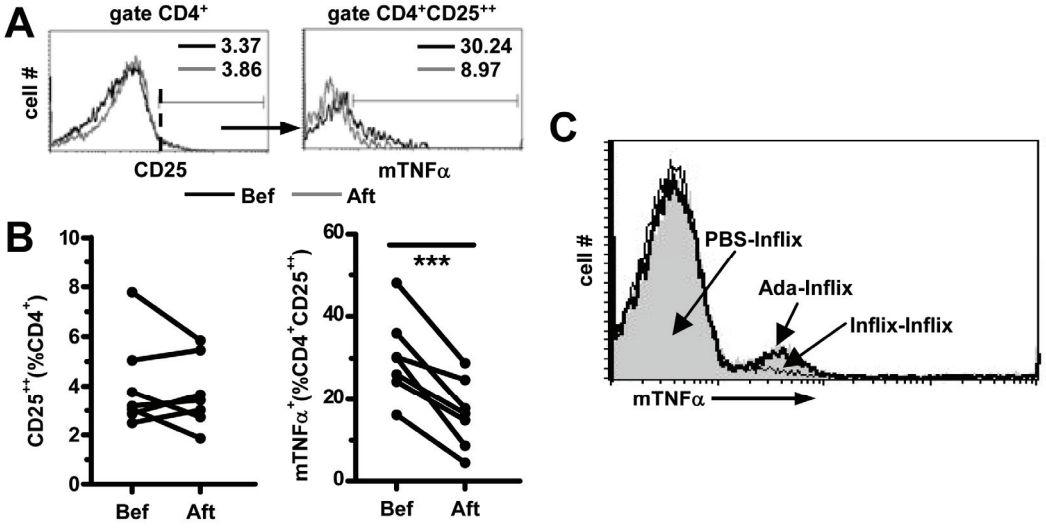

Figure 5. Decreased expression of mTNFα in patients with RA after anti-TNFα treatment. Seven patients with active RA (Disease Activity Score in 28 joints > 5) were evaluated for CD25 and mTNFα expression both before and 3 months after anti-TNFα therapy (40 mg adalimumab subcutaneously every other week in combination with stable doses of methotrexate [7.5–25 mg/week orally]). **(A)** Representative histogram graphs showing CD25 staining on CD4$^+$ T cells (*left panel*) or mTNFα expression on CD4$^+$CD25^{++} T cells (*right panel*) in RA patients before (Bef, black line) and three months after (Aft, gray line) adalimumab treatment. Numbers indicate the percentage of CD25^{++} expression on CD4$^+$ T cells (*left panel*) or mTNFα expression on CD4$^+$CD25^{++} T cells (*right panel*). **(B)** Summary of the percentage of CD25^{++} T cells within CD4$^+$ T cells (*left panel*) or of the frequency of CD4$^+$CD25^{++} T cells co-expressing mTNFα (*right panel*) in RA patients before (Bef) and 3 months after (Aft) TNFα-blocking therapy. Each dot represents one patient (n=7). **(C)** Histogram showing lack of effect of adalimumab on the detection of mTNFα by FITC-labeled infliximab. Cells were incubated with PBS (PBS-Inflix, shaded area), adalimumab (Ada-Inflix, thick black line) or infliximab (Inflix-Inflix, thin black line) at 4˚C for 30 mins. After washing, cells were stained for mTNFα expression by infliximab^{FITC}. *** = $P < 0.001$.

DISCUSSION

Immunosuppressive $CD4^+CD25^+$ Treg cells are of great interest for immunotherapy to prevent transplant rejection and for the treatment of autoimmune diseases. Successful treatment of mice with collagen-induced arthritis, an animal model for human RA, by adoptive transfer of $CD4^+CD25^+$ Treg cells has been reported (9). However, unlike the situation in mice, in which expression of CD25 identifies a relatively homogeneous population of Treg cells, the identification and manipulation of Treg cells in humans is hampered due to the presence of large amounts of activated conventional T cells within the $CD4^+CD25^+$ T cell compartment. Although the highest expression of CD25 (termed $CD25^{high}$ or $CD25^{++}$) on $CD4^+$ T cells is now widely used to identify, isolate, and characterize naturally occurring Treg cells in humans, the reported defective suppressive function of the isolated $CD4^+CD25^{++}$ T cell population from patients with autoimmune diseases, such as RA, might still be attributed, at least in part, to the heterogeneous composition of this T cell population (10-15). Therefore, one of the main obstacles to therapeutic applications of Treg cells is to define and isolate cells displaying high inhibitory activity.

TNFα is a pleiotropic cytokine critical for inflammation, maintenance of secondary lymphoid organ structure, and host defense against various pathogens as well as for the pathogenesis of RA (32-34, 41, 42). Moreover, mTNFα expressed on the surface by $CD4^+$ T cells activates macrophages, costimulates B cell activation, and increases antibody production (27-29). Therefore, we speculated that mTNFα-expressing $CD4^+CD25^{++}$ T cells are enriched for activated conventional T cells and/or T cells with inferior suppressive function, since Treg cells inhibit the function of monocytes/macrophages, reduce antibody production of B cells, and produce very small amounts of TNFα, even in the presence of IL-2 (5, 6, 21). Our results reveal a significant expression of mTNFα within the $CD4^+CD25^{++}$ T cell population in both RA patients and in healthy individuals (Figure 1 and data not shown). Although $mTNF\alpha^+CD4^+CD25^{++}$ T cells display suppressive activity, this activity is much less potent than that of their $mTNF\alpha^-$ counterparts (Figure 4). Therefore, we conclude that mTNFα expression is a marker that is inversely associated with the suppressive capacity of $CD4^+CD25^{++}$ T cells.

It has been shown that surface CD127 expression is inversely correlated with the expression of Foxp3 and suppressive function of fresh human $CD4^+$ T cells (37). However, our data indicate that these two markers are not differentially expressed by $CD4^+CD25^{++}$ T cells that do and those that do not express mTNFα (Figure 2). Although Foxp3 was thought to program the development and function of murine $CD4^+CD25^+$ Treg cells (19), Foxp3 is not a Treg-specific marker in humans, since it is also expressed in activated effector T cells (20-22, 43). Likewise, not all of the $CD4^+CD127^{low/-}$ cells in human PBMCs are $Foxp3^+$, since CD127 is downregulated in recently activated effector T cells (22, 37). Therefore, our data suggest that the expression levels of Foxp3 or CD127 do not correlate with the suppressive activity *within* the $CD4^+CD25^{++}$ T cell compartment, whereas the absence of mTNFα on $CD4^+CD25^{++}$ T cells can be used to characterize and enrich Treg cells with maximal suppressor potency.

Recent reports have suggested that *in vivo* a portion of the human $CD4^+CD25^{++}$ T cell population is generated from rapidly dividing, highly differentiated memory $CD4^+$ T cells (44, 45). Since the memory T cell marker CD45RO is highly expressed on $mTNF\alpha^+CD4^+CD25^{++}$ T cells (Figure 2), and mTNFα expression on these cells correlates with disease activity (Figure 1D), it is tempting to speculate that these Foxp3 positive, anergic $mTNF\alpha^+CD4^+CD25^{++}$ T cells are “adaptive” Treg cells derived from memory T

cells in the periphery. Such cells could emerge as a negative feedback to dampen immune responses, as proposed previously (44-47). Our results showing that a subset of $CD4^+CD25^{++}$ T cells display a less potent suppressive capacity and bear additional activation markers, such as CD45RO, would support this notion. The reason why such cells also express mTNFα could relate to their former function as effector cells, during which they secreted TNFα. The expression of mTNFα would, in that context, be a residual property of these cells. This could also explain the correlation between their presence and disease activity, since it is conceivable that greater disease activity would lead to more exhausted effector T cells that express mTNFα.

Treatment of RA patients with TNFα blockers results in significant clinical benefit, which most likely involves the inhibition of the TNFα-induced inflammatory cytokine cascades (34). Moreover, recent studies have suggested that anti-TNFα antibodies could also reverse the "compromised" suppressive function of isolated $CD4^+CD25^{++}$ T cells in RA patients (10-12, 30). This restoration is thought to involve the neutralization of circulating soluble TNFα that inhibits Treg function *via* TNFRII and/or to induce a distinct $CD62L^-CD25^{++}$ Treg cell population *via* TGF-β in RA patients (11, 12). Considering the fact the adalimumab could deplete mTNFα-expressing cells *via* inducing apoptosis, antibody-dependent cellular cytotoxicity (ADCC), and/or complement-dependent cytotoxicity (CDC) both *in vitro* and *in vivo* (48-50), our results provide an additional explanation for the reversal of Treg cell activity of the isolated $CD4^+CD25^{++}$ T cell population after anti-TNFα antibody therapy, since they indicate that these antibodies deplete the less suppressive $mTNF\alpha^+CD4^+CD25^{++}$ T cells from the $CD4^+CD25^{++}$ T cell population in RA patients (Figure 4 & 5).

In conclusion, we have demonstrated that mTNFα is expressed on a significant percentage of $CD4^+CD25^{++}$ T cells in human peripheral blood, and that this expression is correlated with disease activity in RA patients. Moreover, the absence of surface mTNFα expression can be used to identify and isolate a subset of $CD4^+CD25^{++}$ T cells with potent suppressive capability. Furthermore, our results indicate that, in addition to blocking soluble circulating TNFα and/or inducing a new Treg population (11, 12), selective depletion of the less effective $mTNF\alpha^+$ suppressors from the $CD4^+CD25^{++}$ T cell compartment in RA patients may be another explanation for the recovery of Treg function by anti-TNFα therapy.

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ABBREVIATIONS

mTNFα, membrane-bound TNFα; **Treg**, T regulatory.

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