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Modulation of CD28 Expression With Anti–Tumor Necrosis Factor α Therapy in Rheumatoid Arthritis

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Objective. **The immune system of patients with rheumatoid arthritis (RA) is characterized by the accumulation of CD4+ T cells deficient in CD28 expression and the up-regulation of tumor necrosis factor α (TNFα). Previous in vitro studies have shown that TNFα induces transcriptional silencing of the *CD28* gene. Because reduced expression of CD28 in T cells compromises immunocompetence, we examined whether CD28 expression is reduced in patients with RA in vivo and whether the reduction is related to TNFα.**

Methods. **Patients with RA and age-matched individuals were recruited. Peripheral blood mononuclear cells were stained for CD3, CD4, CD8, CD28, TNF receptor I (TNFRI), and TNFRII, and analyzed by quantitative flow cytometry. The number of CD28 and TNFR molecules was monitored in a subgroup of patients with RA undergoing treatment with anti-TNFα.**

Results. **In addition to higher frequencies of CD28^{null} T cells, patients with RA had significantly reduced numbers of CD28 and TNFRI molecules on CD4+,CD28+ T cells. Normal expression could be restored in vitro by overnight culture, suggesting that CD28 in patients was modulated by exogenous factors. In contrast, treatment with TNFα in vitro resulted in further down-regulation. CD28 expression was normalized in patients undergoing TNFα-neutralizing therapy.**

Conclusion. **Overproduction of TNFα in RA induces a global down-regulation of CD28 in CD4+ T cells and may cause reduced sensitivity to costimulatory signals in T cell responses.**

CD28 is the quintessential costimulatory molecule required for the productive activation, proliferation, and differentiation of effector function in T cells (1). The role of CD28 in cell-mediated immunity is demonstrated by the *CD28* gene–knockout mouse, which has an immunosuppressed phenotype, and CD28$^{-/-}$ mouse T cells are unable to sustain activation and are susceptible to apoptosis (2,3). In various experimental systems, blockade of CD28 interaction with its ligands CD80 and CD86 has been demonstrated to dampen antigen-specific immune responses (4). CD28–CD80/CD86 interaction has therefore been suggested as a target for immunotherapy of chronic inflammatory diseases, such as rheumatoid arthritis (RA) (5,6).

Human T cells have variable levels of CD28 expression. Normal aging is associated with the irreversible loss of CD28 in both the CD4 and the CD8 compartments (7,8), and the frequency of CD28^{null} T cells has been found to be a predictor of nonresponsiveness to influenza vaccination in the elderly (9). Several chronic diseases, such as RA (10), juvenile idiopathic arthritis (11), ankylosing spondylitis (12), Wegener's granulomatosis (13), and Crohn's disease (14), have also been found to be associated with unusually high frequencies of CD28^{null} T cells that are disproportionate with patient age. In RA and Wegener's granulomatosis, the frequency of CD4+,CD28^{null} T cells is correlated

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with the severity of clinical disease manifestations (15,16).

Unlike the findings in *CD28*-knockout mice, human $CD28^{null}$ T cells are functionally active. They are highly oligoclonal, long-lived lymphocytes with aberrant functions that are thought to contribute to disease-related immune dysfunctions (17). CD4+,$CD28^{null}$ T cells have lost their classic helper function because of an accompanying inability to up-regulate CD40 ligand (18), but they have acquired granzyme and perforin that impart cytotoxicity (19). They also have large cytoplasmic stores of interferon-γ (20), express the chemokine receptor CCR5 (21), and up-regulate CD161 (22), a molecule that facilitates tissue invasion. Hence, CD4+,$CD28^{null}$ T cells are equipped to infiltrate sites of inflammation, where they amplify local inflammatory and autoimmune cascades (22,23).

Because the loss of CD28 expression is coupled with functional aberrations, biologic situations in which CD28 expression is modulated have been of significant interest. T cell activation has been shown to invariably result in the down-regulation of CD28 (24,25). In addition, exposure of CD28+ T cells to tumor necrosis factor α (TNFα) results in the emergence of $CD28^{null}$ progeny (26) due to TNFα-induced inhibition of *CD28* gene transcription (27). Long-term exposure of T cells to TNFα could therefore accelerate the development and accumulation of $CD28^{null}$ T cells in vivo. Since TNFα is considered to be the dominant inflammatory cytokine in the pathogenesis of RA (28), we examined quantitative changes in the cell surface expression of CD28 on CD28+ T cells and how they relate to the clinical responses of patients undergoing anti-TNFα therapy.

PATIENTS AND METHODS

Study population. Patients with RA (age range 18–88 years; median age 55 years; female:male ratio 3:1) and age-matched healthy individuals (age range 21–85 years; median age 58 years; female:male ratio 2:1) were enrolled. All patients with RA met the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for the diagnosis of RA (29), and 85% were diagnosed as having erosive disease. In a subgroup of RA patients eligible to receive anti-TNFα treatment with infliximab (Centocor, Malvern, PA), blood was obtained before and 1 week after the first treatment. Clinical responses of patients to therapy were independently monitored by the treating physician, using the ACR criteria for treatment response (30). All study participants provided written informed consent. Personal and clinical information and biologic specimens were coded. All protocols involving human subjects were approved by the institutional review boards of the Mayo Foundation, Emory University, and the University of Pittsburgh.

Isolation of mononuclear cells and cell culture. Peripheral blood mononuclear cells (PBMCs) were isolated by standard isopycnic centrifugation over Ficoll-Hypaque gradient. PBMCs were cultured at a density of 1×10^6/ml in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal calf serum (Summit Biotechnology, Fort Collins, CO), 2 m*M* L-glutamine, 50 units/ml penicillin, and 5 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cultures were incubated for 48 hours with 10 ng/ml recombinant human TNFα (R&D Systems, Minneapolis, MN). This concentration of TNFα had been previously found to be nontoxic, and optimally down-regulated CD28 expression on T cells (26).

Flow cytometry. Freshly isolated or cultured PBMCs were stained with a combination of either phycoerythrin (PE)–conjugated anti-CD28, PE-conjugated anti–TNF receptor I (anti-TNFRI), or PE-conjugated anti-TNFRII and fluorescein isothiocyanate (FITC)–conjugated anti-CD4 (or anti-CD8) and peridin chlorophyll protein–conjugated anti-CD3 (Becton Dickinson, San Diego, CA). Raw cytometric data were acquired immediately after staining using a FACScan flow cytometer with CellQuest and QuantiQuest software (Becton Dickinson). Data acquisition in all flow cytometry experiments was performed using the same instrument at identical flow and electronic settings. Analyses of cell populations were performed using WinMDI software (J. Trotter, Scripps Research Institute, La Jolla, CA).

The number of CD28, TNFRI, and TNFRII molecules was measured by quantitative flow cytometry using the QuantiBrite system (BD Biosciences, San Jose, CA), as previously described (26). Cytometric data for cells stained with each of the PE-conjugated antibodies and 4 QuantiBrite-PE bead standards of known levels of PE fluorescence were acquired using QuantiQuest software. Raw data were obtained with a single flow cytometer, and each cytometric analysis was performed at identical instrument settings. The fluorescence channel 2 axis was transformed as the number of bound PE molecules per cell and was calibrated against the QuantiBrite beads. Slope and intercept information from the regression analysis was automatically saved with the acquired raw data files. The actual number of CD28, TNFRI, or TNFRII molecules per cell was estimated with QuantiCALC software (Verity Software House, Topsham, ME).

Statistical analysis. Quantitative data were subjected to statistical analysis using SigmaStat software (SPSS, Chicago, IL). Two-group comparisons were assessed by Student's *t*-test or by the Mann-Whitney U test, if appropriate. Paired *t*-tests were conducted for measurements of paired samples. For regression analysis, regression lines were calculated within a 99% confidence interval. *P* values less than 0.05 were considered significant.

RESULTS

Down-regulation of CD28 in RA. Consistent with previous studies (10,15), patients with RA showed an increased frequency of $CD28^{null}$ T cells in the CD4 compartment. We also documented the abundance of these cells in the CD8 compartment (Figure 1A). $CD28^{null}$ T cells were found only sporadically among CD4 T cells in age-matched controls ($P < 0.0001$). $CD28^{null}$ T cells were

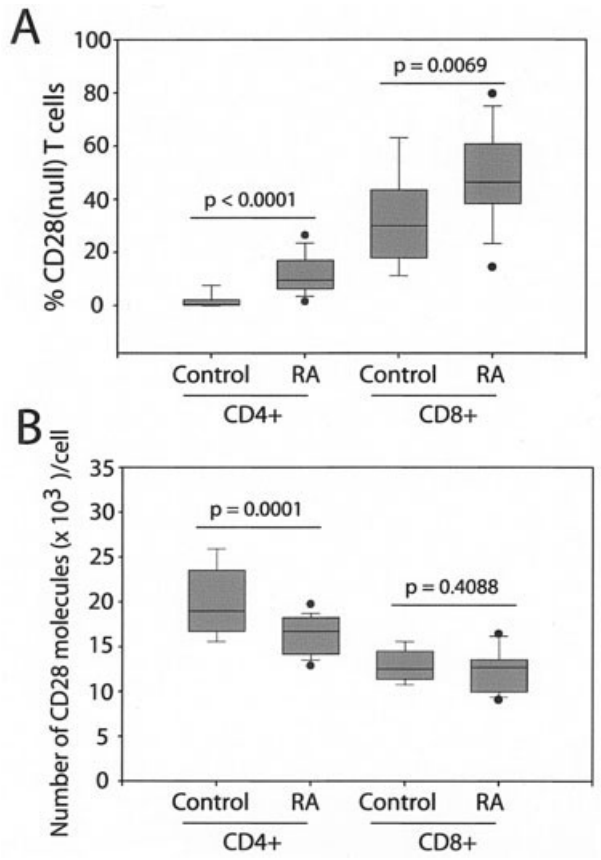

Figure 1. CD28 expression on T cells in rheumatoid arthritis (RA) patients. Peripheral blood mononuclear cells from patients with RA and age-matched controls were immunostained for CD3, CD4, CD8, and CD28, and analyzed by flow cytometry. **A,** The frequency of CD3+,CD28^{null} T cells in the CD4 and CD8 compartments was measured (n = 17 controls and 26 RA patients). **B,** The number of CD28 molecules on the surface of CD3+,CD28+ T cells was determined by quantitative flow cytometry (n = 18 controls and 29 RA patients). All data shown were analyzed by nonparametric testing. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles.

present in the CD8 compartment of healthy individuals, which is consistent with previous studies (7). However, patients with RA had significantly increased frequencies of CD8+,CD28^{null} T cells ($P = 0.007$).

In addition, patients with RA had significantly lower numbers of CD28 molecules on the surface of CD4+,CD28+ T cells compared with age-matched healthy controls ($P = 0.0001$) (Figure 1B). These patients were taking a variety of disease-modifying antirheumatic drugs, mostly methotrexate, but were not taking anti-TNFα agents. There was no obvious correlation between CD28 density and treatment. In contrast to CD4+ T cells, the number of CD28 molecules on CD8+,CD28+ T cells was comparable between patients and controls. It should be noted, however, that CD28 expression levels on CD8+ T cells were significantly lower than on CD4+ T cells in both groups, a finding consistent with those of previous studies (25,31). Because of these findings, subsequent measurements of CD28 density were focused on CD4+ T cells.

Correlation of CD28 levels and TNFR expression. TNFα is known to down-regulate its receptors, TNFRI and TNFRII (32,33). Hence, the level of TNFR expression is considered to be indicative, if not predictive, of the amount of bioactive TNFα that cells are exposed to in vivo (33). Because CD28 is sensitive to down-regulation by TNFα (26), we examined whether the levels of CD28 and TNFR expression were correlated. Figure 2 shows that the number of CD28 mole-

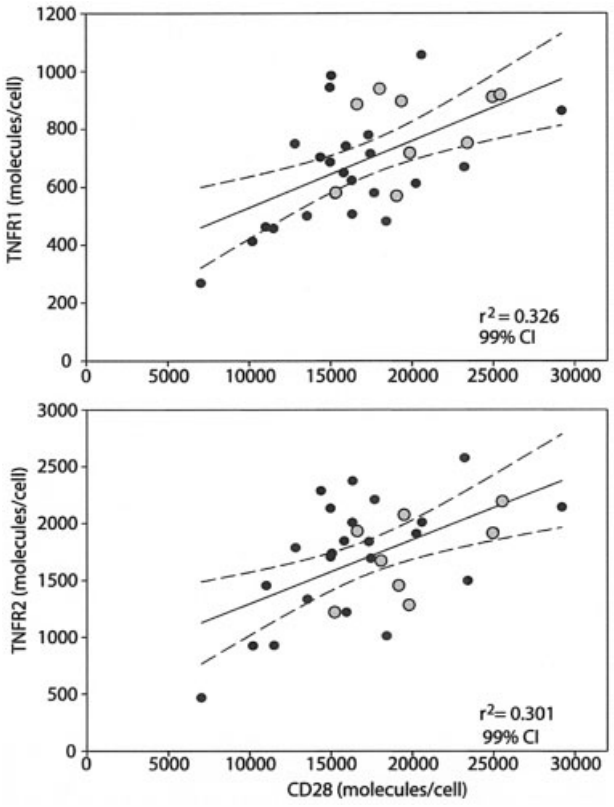

Figure 2. Correlation between cell surface expression of CD28 and tumor necrosis factor receptor (TNFR) levels. Quantitative flow cytometric assays of CD28, TNFRI, and TNFRII were performed on the same day. The values were plotted and regression analysis was performed. Data shown include the calculated regression (solid lines) ($P < 0.05$) and the 99% confidence intervals (99% CI) (broken lines). n = 23 rheumatoid arthritis patients (solid circles) and 9 age-matched controls (shaded circles).

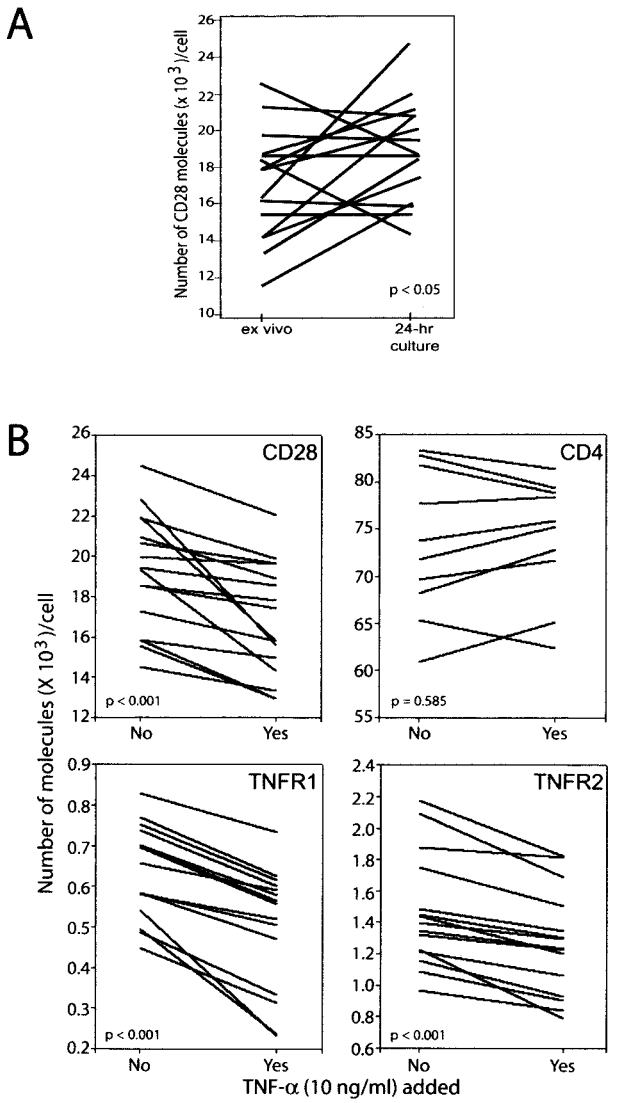

Figure 3. Modulation of CD28 and tumor necrosis factor receptor (TNFR) expression in vitro. **A,** Peripheral blood mononuclear cells (PBMCs) from patients with rheumatoid arthritis (n = 15) were isolated (ex vivo), and aliquots were cultured for 24 hours without stimulation or exogenous growth factors. **B,** A parallel set of PBMCs (n = 16) was cultured for 48 hours in the presence or absence of recombinant TNFα. In both experiments, the number of CD4, CD28, TNFRI, and TNFRII molecules on CD3+ T cells was measured by quantitative flow cytometry. Data shown are pairwise comparisons for each patient examined. Statistical significance was analyzed by paired *t*-test.

cules on T cells was directly proportional to the number of TNFRI and TNFRII molecules. CD28 and TNFRI expression was correlated at $r^2 = 0.326$; CD28 and TNFRII expression was correlated at $r^2 = 0.301$. Compared with healthy individuals, RA patients tended to have lower expression levels of TNFRI ($P < 0.05$), but not TNFRII. When regression analysis was performed by excluding the values from healthy donors, similar correlations between CD28 and TNFR densities were observed.

Expression of CD28 and TNFR subject to modulation. To examine whether low levels of expression of CD28 and TNFRI in patients with RA were due to exogenous factors and were reversible, expression of CD28 and TNFR in isolated PBMCs was monitored over time. Figure 3A shows that overnight culture of PBMCs in medium alone with no stimulation or with exogenous growth factors resulted in the modulation of CD28 expression on CD4+ T cells. Although there was individual variability in the degree of modulation, the patient cohort examined showed significant increases ($P < 0.05$ by paired *t*-test) in the number of CD28 molecules postculture over the levels seen in immediate ex vivo analysis. A nearly superimposable pattern of increased expression of TNFRI and TNFRII was also observed (data not shown).

Since RA is associated with the up-regulation of TNFα (28), the effect of TNFα in the in vitro culture system was also studied. PBMCs were cultured for 48 hours in the presence or absence of TNFα without additional stimulation or other exogenous growth factors. As shown in Figure 3B, TNFα induced the down-regulation of CD28, TNFRI, and TNFRII on CD4+ T cells in vitro ($P < 0.001$ compared with unstimulated cultures). The down-regulation of these cell surface molecules was observed in all patients examined. In contrast, CD4 molecules themselves were not significantly affected by TNFα.

Up-regulation of CD28 and TNFRI expression elicited by anti-TNFα therapy. The above results showed that CD28 and TNFR are clearly sensitive to modulation by TNFα. If the physiologic environment of RA is TNFα-rich (28), then with increased levels of neutralizing antibodies to TNFα, the levels of CD28 and TNFR expression would be expected to change. Figure 4 shows that CD28 levels were altered by 1 week of anti-TNFα therapy. The majority of the patients examined (11 of 16 [68.8%]) showed increased numbers, with the entire group showing a statistically significant increase in the number of CD28 molecules ($P < 0.05$ by paired *t*-test). Up-regulation was associated with a measurable improvement in the patients' clinical disease activity, with 9 of 16 patients (56.3%) meeting the ACR 20% improvement criteria (ACR20) or better. Interestingly, 4 of the 5 patients who did not have an increase in

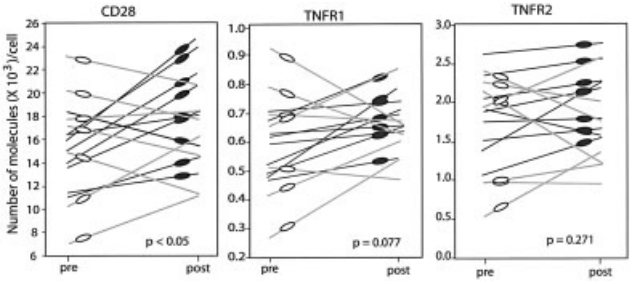

Figure 4. Increased expression of CD28 and tumor necrosis factor receptor I (TNFRI) after treatment with anti-TNFα. Sixteen patients with rheumatoid arthritis were monitored for levels of CD28 and TNFR expression on CD3+,CD4+ T cells before treatment and 1 week after the first infusion of infliximab. Data shown are pairwise comparisons for each patient examined. Solid symbols represent patients who responded to therapy (measured by the American College of Rheumatology 20% improvement criteria); open symbols represent patients who did not respond to therapy.

CD28 surface expression also did not achieve an ACR20 response.

There was a similar pattern of increased TNFRI numbers 1 week after therapy, but the change did not reach statistical significance ($P = 0.08$ by paired t-test) and did not appear to correlate with treatment response. The levels of TNFRII were not affected by therapy.

To ascertain the validity of these results, additional cross-sectional cohorts of patients not receiving anti-TNFα treatment and patients who had been receiving anti-TNFα treatment for 1 week or 3 months were examined. Figure 5 shows that there was a steady and significant increase in the number of CD28 molecules during the 3 months of therapy. Similarly, TNFRI

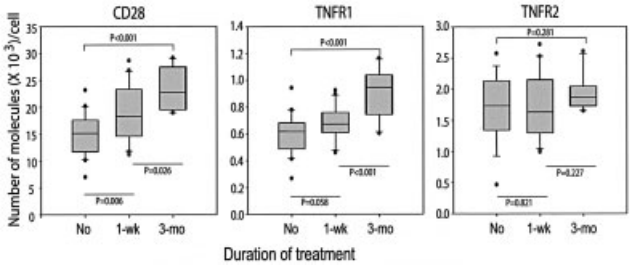

Figure 5. Recovery of expression of CD28 and TNFRI in patients receiving long-term anti-TNFα treatment. PBMCs in patients with rheumatoid arthritis who were not receiving TNFα blockers (n = 20) or had been treated with infliximab for 1 week (n = 20) or 3 months (n = 10) were analyzed for CD28, TNFRI, and TNFRII expression on CD3+,CD4+ T cells. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and the 90th percentiles. See Figure 3 for definitions.

numbers also increased. In this cross-sectional study, an increase in TNFRI numbers was observed after only 1 week of therapy but was not statistically significant when compared with the untreated group, a result that recapitulated the longitudinal measurements in Figure 4. However, there was a significant increase in TNFRI numbers within 3 months. In contrast, TNFRII numbers did not show significant changes within the 3 months of therapy. It should be noted that all patients receiving anti-TNFα therapy showed clinical improvement, with an ACR20 response or better.

DISCUSSION

RA is associated with large clonal expansions of T cells that are present in the peripheral circulation and are not only restricted to the inflamed synovium (34). Studies from various laboratories have consistently demonstrated that these oligoclonal T cells are deficient in expression of the CD28 costimulatory receptor (17,35–37). CD4+,CD28^{null} T cells are long-lived, functionally aberrant lymphocytes (38). They have a myriad of loss-of-function and gain-of-function properties through which they contribute to the pathogenesis of the disease. Direct involvement of these cells in RA would also explain why the severity of the clinical disease is correlated with the frequency of CD4+,CD28^{null} T cells (15,36). The increased oligoclonality of the memory T cell repertoire has been presumed to be the reason for the increased frequencies of CD4+ and CD8+,CD28^{null} T cells in RA patients compared with age-matched controls (Figure 1). Here, we show that the number of CD28 molecules is also reduced significantly in the CD28+ T cell subset among patients, documenting that RA patients have a global deficiency in CD28 costimulatory function that is not explained by the long-term activation of a few autoreactive T cells. This deficit appears to be, at least in part, reversible (Figures 3–5) and related to the overproduction of TNFα.

CD28 is normally subject to modulation following T cell activation (24,25), and down-regulation and/or irreversible loss of CD28 expression on T cells in patients could indicate chronic immune activation in RA (38). This suggestion is consistent with studies demonstrating that repeated stimulation of T cells in vitro results in the progressive down-regulation of CD28 and, eventually, the emergence of CD28^{null} T cells (17,25,31). Previous studies have also demonstrated that the loss of CD28 on T cells is accelerated by TNFα (26), the cytokine that is thought to be the dominant amplifier of

local inflammation in the rheumatoid synovium and/or modifier of systemic complications in RA (28).

Consistent with previous findings showing a direct repressive effect of TNFα on *CD28* gene transcription (26,27), we found that exposure of patient-derived T cells to TNFα in vitro consistently resulted in the further down-regulation of CD28 (Figure 3B), showing that T cells in RA patients are responsive to TNFα. Consistent with previous reports that TNFα down-regulates expression of its own receptors (33,39), our data show that T cells have decreased numbers of TNFRI and TNFRII molecules that correspond with a quantitative decrease of CD28 molecules in response to TNFα in vitro (Figure 3B). We also found concordance between expression levels of CD28 and TNFR in vivo (Figure 2), thereby indirectly implicating TNFα in the down-regulation of CD28.

The basis for the dichotomy of TNFR expression in vivo, i.e., TNFRI, but not TNFRII, expression was found to be significantly reduced among patients with RA, is unknown. Previous studies have shown that TNFRI appears to be subject to receptor shedding, whereas TNFRII is selectively endocytosed in response to TNFα (32,33,39). Whether TNFRI and TNFRII are subject to differential regulation in RA is also not known, but our data indicate that both receptors are functional among patients and are equivalently subjected to modulation by TNFα in vitro (Figure 3B). Both receptors have been previously indicated to elicit CD28 down-modulation (26), although the TNFα/TNFR signaling pathway leading to CD28 down-regulation remains to be elucidated.

Consistent with the notion that the physiologic in vivo environment of RA is TNFα-rich (28) and promotes CD28 down-regulation (26), our data also show significant up-regulation of CD28 when T cells are placed into tissue culture without additional stimulation or exogenous growth factors (Figure 3A). The reason for the individual variability in the degree of postculture CD28 modulation is not clear at this time. However, TNFα is known to interact with mannose diesters of glycan moieties from membrane proteins (40), a biochemical property of many cytokines that promotes the extension of cytokine biologic half-life (41,42). Hence, it is possible that the observed down-regulation, versus up-regulation, of CD28 on the T cells in some patients (even after overnight culture) (Figure 3A) could be instances when TNFα became sequestered in the T cell membrane glycocalyx and remained biologically active for some period of time. An alternative interpretation is that TNFα is not uniformly up-regulated in RA patients.

We have previously reported that the loss of CD28 is only partially reversible (43) and that the frequency of $CD28^{null}$ T cells is associated with the propensity of patients to develop severe forms of RA (15,36). The dynamic changes in CD28 expression on CD4+ T cells have also been shown by other investigators to correlate with the clinical course of disease activity (44). Therefore, if such changes in the in vivo expression levels of CD28 are indeed coupled with the dysregulation of TNFα in RA (26–28), then anti-TNFα therapy (45–47) could also have an impact on CD28 expression. Our data show a significant trend toward an increase in the number of CD28 molecules in patients posttreatment (Figure 4). Interestingly, individual patients who were nonresponsive to therapy also tended to not have an increase in the expression levels of CD28 and TNFRI molecules.

In view of the sensitivity of CD28 and TNFRI to down-regulation by TNFα (26,27,32,33) (Figure 3B), the combined cellular and clinical outcomes indicate a lack of increased TNFα production or the limited neutralization of bioactive TNFα by the infusion of anti-TNFα antibodies. In cross-sectional studies of patients receiving anti-TNFα treatment, we found continuous normalization in the numbers of CD28 and TNFRI molecules. Within 3 months of the start of therapy (Figure 5), the recovery of CD28 and TNFRI expression on T cells in patients was such that they reached levels comparable with those of healthy controls (Figure 1). These data are consistent with the idea that the degree of down-regulation of CD28 expression is indicative of the clinical course of RA (15,38,44). In light of the plethora of clinical outcome measures that have wide margins of variability in ascertaining patient response to anti-TNFα therapy (45–47), our data indicate the value of obtaining the quantitative levels of CD28 induction for use as a convenient biomarker for the clinical outcome of therapy (Figures 4 and 5).

Since CD28 is central to the induction of immune responses (4), and the loss or lack of CD28 is associated with functional aberrations of T cells (17,38), the globally decreased expression of CD28 on T cells in RA patients should have functional implications for the immune competence of these patients. It is, however, difficult to distinguish the impact of low CD28 expression from other variables. Data on the immune competence of RA patients, such as the information garnered from vaccine responses or by tumor surveillance, are incomplete. Even when known, they are difficult to interpret. Patients with RA have several reasons to be immunocompromised, including having a prematurely

aged immune system, reduced thymic function, and accelerated telomere shortening of peripheral T cells, in addition to undergoing immunosuppressive treatment interventions (48).

It remains to be investigated whether induction of CD28 during anti-TNFα therapy (Figures 4 and 5) translates to the improvement of immune competence in the adaptive immune system. We previously reported that restoration of CD28 expression in vitro for certain $CD28^{null}$ T cells can also reverse an accompanying deficiency in CD154 expression and restore the helper function of these cells (43). Nonetheless, there is increasing evidence that innate defenses can be compromised with anti-TNFα therapy (49). The recent finding that it is therapeutically beneficial to use CTLA-4Ig to block the CD28–CD80/CD86 interaction (50) adds complexity and raises the possibility that these 2 treatment interventions, with CTLA-4Ig and with TNFα-neutralizing agents, are at least partially antagonistic.

It might be noted that in addition to CD28, the T cell receptor (TCR) has also been found to be greatly affected by TNFα. Exposure of T cells in vitro to TNFα has been shown to down-regulate expression of CD3ζ, one of the signaling subunits of the TCR/CD3 complex (51). TNFα can also inhibit the phosphorylation of CD3ζ, thereby attenuating the TCR signaling cascade (52). These TNFα-mediated deficits of TCR function are reversible in a manner similar to that seen in the present work, with the reversibility of TNFα-induced down-regulation of CD28 expression (Figures 3–5). Since productive activation of T cells requires the coengagement of the TCR/CD3 complex and the CD28 costimulatory receptor (1,4), these studies suggest that comodulation of CD3ζ and CD28 by TNFα likely contributes to T cell dysfunction in the TNFα-rich environment of RA (38). The reversibility of the downmodulatory effects of TNFα on CD3ζ and CD28 suggests that an ultimate benefit of TNFα neutralization therapy in RA might be the enhancement of immune competence. Testing this hypothesis will require long-term prospective analysis of immune functions in patients undergoing this type of therapy, which consistently shows measurable clinical benefits (45–47).

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