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Unchecked CD70 Expression on T Cells Lowers Threshold for T Cell Activation in Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a systemic inflammatory disease that predominantly manifests in diarthrodial joints, leading to structural damage of the joint architecture (1, 2). The main effector mechanisms causing cartilage degeneration and bony erosion include hyperplasia of synoviocytes; production of various cytokines, in particular TNF-α; and receptor activator of NFκB ligand-mediated activation of osteoclasts (2–4). The nature of the defect upstream of these effector functions is a matter of debate (1, 5). In one model, the inflammation is considered to be the result of a misguided T cell response. Several lines of indirect evidence have supported this notion (2, 6). The major disease-associated genetic risk factor is an HLA-DRB1 polymorphism that is important for the function of CD4+ T cells (7, 8). Also, newly defined genetic risk factors, such as CLTA-4 and PTPN22, are concerned with T cell physiology (9, 10). Experiments in rheumatoid synovium/SCID mouse chimera animal models showed that synovial inflammation is strictly T cell dependent (11). Moreover, many patients show evidence of lymphoid neogenesis in the synovium, which optimizes the recognition of Ag by B and T cells (2, 12). Finally, targeting the adaptive immune response has shown therapeutic benefits. The most obvious example is treatment with CTLA4-Ig, which is effective in RA (13). Also, the success of B cell depletion in the treatment of RA has been attributed to the Ag-presenting function of B cells that take up and present Ag to tissue-infiltrating T cells (14).

T cell activation is ultimately determined by positive signals from costimulatory molecules and negative signals from inhibitory receptors that are expressed on T cells and bind to ligands on APCs (15–18). The classical example for such a costimulatory pathway is the CD28-CD80/CD86 interaction, which is particularly important in activating naive T cells (15). Indeed, treatment with CTLA4-Ig that interferes with this receptor-ligand interaction reduces joint inflammation in RA (13). Another costimulatory pathway important for the activation of naive T cells is the stimulation of CD27 that recognizes the CD70 molecule (16, 18–20). CD28 and CD27 are constitutively expressed, and costimulatory activities are controlled by the expression of the ligands CD80/CD86 and CD70, all of which are tissue specific and spatially and temporally restricted (16, 19, 20).

In addition to CD28 and CD27, which mostly control the activation of naive CD4 T cells, a large array of molecules has been shown to regulate T cell activation. One major variable in determining the profile of regulatory molecules on T cells is the age of the individual, or, possibly better stated, the replicative history of the T cell population (2, 21). Many of the receptors that have been found to control the function of senescent or end-differentiated T cells are primarily expressed on NK cells. Such molecules include members of the killer Ig-like receptor family, NKGD2, fractalkine receptors, and Ig-like transcripts (21–24). In contrast, regulatory molecules that are usually associated with T cell function, such as CD28, CD27, and CD40L, are frequently lost in such T cells (21). These changes appear to be of particular importance for the pathogenesis of RA, a disease of late adulthood with increasing age-related incidence (2). Moreover, the adaptive immune system in RA patients is preaged with premature appearance of many of these T cell senescence markers as compared with age-matched healthy controls (2, 6, 21). Indeed, many of these aberrantly expressed T cell regulatory molecules appear to be functionally important in RA and synovial inflammation. Killer Ig-like receptor 2DS2 has been shown to be a genetic risk factor for extra-articular complications of RA (2, 25). NKGD2 is expressed on end-differentiated CD4 T cells and provides a costimulatory signal recognizing a ligand expressed in the synovial tissue (22, 26). Similarly, fractalkine receptor is expressed on senescent CD4 T cells and communicates with synovial fibroblasts through
the recognition of fractalkine (23). Aberrant expression of regulatory molecules on T cells may, therefore, be an important component enabling the rheumatic disease process.

We hypothesized in this study that comparing the gene expression profiles of CD4+CD28+ and CD4+CD28− T cells from patients would allow for identifying molecules related to accelerated immune aging in RA and involved in RA pathogenesis. The most striking difference in cell surface molecules identified in the arrays was the overexpression of the CD27 ligand CD70. Data presented in this study show that CD4+CD28− T cells have a defect in down-regulating CD70, which leads to sustained expression after T cell activation. Accordingly, expression of this molecule is increased on peripheral CD4 T cells from RA patients. Aberrant CD70 expression on bystander T cells lowers the TCR threshold necessary for the induction of primary T cell responses, possibly leading to activation of self-reactive T cells and breaches in tolerance. Therapeutic interventions targeting the expression of CD70 and the CD27-CD70 interaction may, therefore, be of particular benefit for RA patients.

Materials and Methods

Subjects

PBMCs were obtained from 22 patients with rheumatoid factor-positive RA, aged 23–77 years, and 27 healthy volunteers, aged 25–81 years. The protocol was approved by the Mayo Clinic and the Emory University Institutional Review Boards, and all participants provided informed consent.

Individuals with cancer, a history of chemotherapy, advanced atherosclerotic disease or congestive heart failure, poorly controlled diabetes mellitus, or chronic obstructive pulmonary disease were excluded; healthy volunteers did not have a chronic inflammatory disease.

Cell separation

CD4+CD28+ and CD4+CD28− T cells were purified from PBMCs by FACSVantage (BD Biosciences). Cells were stimulated with immobilized DNA methyltransferase (DNMT) control (28) was inhibited with PD98059 in parallel experiments, the ERK pathway that had been shown to be involved in CD70 expression was activated. Accordingly, expression of this molecule is increased on peripheral CD4 T cells from RA patients. Aberrant CD70 expression on bystander T cells lowers the TCR threshold necessary for the induction of primary T cell responses, possibly leading to activation of self-reactive T cells and breaches in tolerance.

Microarray analysis

Total RNA was extracted from CD4+CD28+ and CD4+CD28− T cell lines using a RNeasy Mini Kit (Qiagen). Preparation of biotinylated target RNA from total RNA and subsequent hybridization of cRNA to the Affymetrix Hu-95Av2 (Affymetrix) probe-array cartridges were performed by the Mayo Cancer Microarray Core Facility (Mayo Foundation).

FACS analysis

PBMCs or T cell lines were stained with FITC- or PE-conjugated anti-CD70, FITC or PE anti-CD28, FITC anti-CD69, PerCP anti-CD4, allophycocyanin anti-CD3, PE or allophycocyanin anti-CD28, or allophycocyanin anti-CD25 (all from BD Biosciences), and PE anti-TCR-Vβ2, PE or allophycocyanin anti-CD28, or allophycocyanin anti-CD27. PBMCs or T cell lines were stained with FITC- or PE-conjugated anti-CD45RA magnetic beads (Miltenyi Biotec). In some experiments, CD4 T cells were negatively selected by a CD4+CD45RO− naïve T cell subset column kit (R&D Systems). Naive CD4 T cells were isolated by positive selection with anti-CD45RA magnetic beads (Miltenyi Biotec). In selected experiments, 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma-Aldrich) at a final concentration of 1 μM or zebularine (10 μM; Sigma-Aldrich) was added on day 3 to inhibit DNA methylation. In other experiments, the CD4 T cell lines were stimulated with immobilized anti-CD3 (1 μg/ml) and CD28 (0.5 μg/ml) in the presence of CD70-expressing CD4+CD28+ or control CD4+CD28− T cells; second, CFSE-labeled CD4 naïve T cells were cocultured with mature DC and pulsed with toxic shock syndrome toxin (TSST)-1 (Toxin Technology) in the presence or absence of CD4+CD28− T cells. Proliferation was assessed by CFSE dilution analysis using flow cytometry. In both assay systems, CD70 was blocked by 1 μg/ml anti-CD70 mAb (Ki-24) or isotype control mAb (J606; both from BD Biosciences).

Statistical analysis

Data were analyzed using a nonparametric Mann-Whitney U test (SigmaStat; SPSS).

Results

Overexpression of CD70 on CD4+CD28− T cells

Previous studies have demonstrated that transcriptional inactivation of CD28 on CD4 T cells is closely correlated with a gain of
new regulatory cell surface molecules (21, 26). We and others have proposed that costimulatory signals delivered through aberrantly expressed stimulatory receptors perturb peripheral tolerance and sustain autoimmune responses (22, 23). Aberrantly expressed molecules have to date been identified using a candidate gene approach. To more systematically screen for changes in gene expression of potential regulatory receptors, we compared gene expression profiles of CD4\(^+\)CD28\(^-\) and CD4\(^+\)CD28\(^+\) short-term T cell lines from three patients using Affymetrix microarrays. Among regulatory cell surface molecules, differential gene expression was most impressive for the CD27-CD70 receptor-ligand pair. CD70 was markedly up-regulated in all CD4\(^+\)CD28\(^-\) T cells as compared with in CD4\(^+\)CD28\(^+\) T cells from the same donor. In contrast, CD27 transcripts were barely detected in the CD4\(^+\)CD28\(^-\) T cell lines (Fig. 1A). These data were verified at the protein level by multicolor flow cytometry using CD4 T cell lines that included CD28-negative and -positive T cells. As shown in Fig. 1B, CD4\(^+\)CD28\(^-\) T cells were homogenous for the CD70/CD27 phenotype, whereas CD4\(^+\)CD28\(^+\) T cells were heterogeneous; ~50% were CD27 positive; and the majority CD70 negative. CD70 is
known to be an inducible molecule that is transiently up-regulated by T cells upon stimulation. To exclude that CD4+CD28− cells differ from CD4+CD28+ T cells in their activation state, cells were stained for the expression of CD69, a T cell-activating marker. CD69 was expressed on a minority of T cells and on fewer CD4+CD28− than CD4+CD28+ T cells, indicating that overexpression of CD70 was not related to differential activation (Fig. 1B).

**Persistent CD70 expression on CD4+CD28− T cells after T cell activation**

Although CD70 is clearly overexpressed on cultured CD4+CD28− T cell lines, and the expression is not lost with resting for 2–4 wk after activation, CD70 is only expressed on a fraction of peripheral blood CD4+CD28− T cells (data not shown). We therefore hypothesized that CD4+CD28− and CD4+CD28+ T cells differ in the regulation of CD70 expression after stimulation. To address this question, PBMCs from patients who were known to have increased frequencies of CD4+CD28− T cells were stimulated, and CD70 expressions on T cell subsets were monitored by multicolor flow cytometry. As shown in Fig. 2, expression of CD70 was progressively gained on CD4+CD28− T cells for the first 5 days after stimulation and sustained throughout the 14-day culture. In contrast, only a subset of CD4+CD28+ T cells gained CD70 expression after stimulation, and even these cells started to revert and lose it after 1 wk, which is consistent with the current paradigm that the window of CD70 expression is tightly controlled. These data indicate that the defect in CD70 regulation lies in sustained expression after activation.

**Correlation between CD70 promoter demethylation and CD70 transcription**

To examine whether CD4+CD28+ and CD4+CD28− T cells differ in epigenetic control of CD70, we examined DNA methylation patterns. The CD70 promoter has 20 CpG dinucleotides between −9 and −512 bp. Bisulfite sequencing results shown in Fig. 3A did not support the hypothesis that CpG islands in CD4+CD28− T cells are more demethylated than CD4+CD28+ T cells. Almost all CpG dinucleotides within the proximal promoter up to position −167 were unmethylated in either cell type, whereas the remainder of the promoter was mostly methylated. These results are in contrast to patients with systemic lupus erythematosus (SLE), in which CD70 overexpression has been correlated with demethylation within the region between −515 and −423 (28).

To examine whether the sustained expression of CD70 is caused by progressive promoter demethylation of CD4+CD28− T cells upon activation and failure to remethylate, we examined promoters on day 4 after stimulation. Results did not demonstrate a significant difference between the two cell types. Overall activation-induced demethylation was minimal for CD4+CD28− and CD4+CD28+ T cells (only at positions −338, −383, and −423) (Fig. 3B).

To further determine whether the sustained CD70 expression is due to a failure of CD4+CD28− T cells to remethylate, cells were activated, grown in the presence of the DNMT inhibitor 5-Aza-dC, and examined for CD70 expression. The response pattern was very similar for both T cell types (Fig. 3C). CD4+CD28− T cells had a higher and more sustained CD70 expression compared with CD4+CD28− T cells, which is consistent with the data shown in Fig. 2. Similar results were obtained with a second inhibitor, zebularine. DNMT inhibition increased CD70 expression in both cell types; however, the relative difference between CD4+CD28− and CD4+CD28+ T cells was maintained. In contrast, inhibition of the ERK pathway, which has recently been implicated in modifying methylation patterns of CD4 T cells in SLE patients (29), did not have any effects.

**Increased frequencies of CD4+CD70+ T cells in RA patients**

CD28 loss on memory T cells is a typical sign of immune aging, but is also disproportionately seen in several autoimmune diseases (2, 21). In RA, the frequency of CD4+CD28− T cells predicts severity of erosive disease and occurrence of extra-articular disease (30), suggesting that these cells are involved in disease pathogenesis. To examine whether the phenotypic changes in RA include increased CD70 expression on CD4 T cells, we compared 27
Healthy individuals and 22 RA patients (Fig. 4). CD70 was expressed on CD4+CD28− T cells and to a much lesser extent on CD4+CD28+ T cells (data not shown). The frequencies of CD4+ CD70+ T cells in healthy individuals age 50 years and younger were low; these cells accounted for ~1% of all CD4 T cells. After the age of 50, frequencies increased ~2-fold (p = 0.009). In contrast, RA patients had 4- to 6-fold higher frequencies of these cells compared with age-matched controls (p = 0.004 and p = 0.005, respectively, for RA patients age 50 years and younger or over 50 years). In RA patients, a trend for age-dependent CD70 expression was not significant (p = 0.3).

**CD70 on CD4+CD28− T cells acts as a bystander costimulatory signal**

To explore the physiological implication of sustained CD70 expression, we tested the hypothesis that CD70 provides a costimulatory signal even if it is not expressed on the APC, but on a third-party T cell that provides a bystander signal. CD4+CD28− and CD4+CD28+ T cell lines were established from a patient with RA. Subsequently, PBMCs were obtained from the same patient whose disease was controlled at that time on weekly methotrexate, labeled with CFSE, and activated with immobilized anti-CD3 in the presence of either autologous CD4+CD28− or CD4+CD28+ T cells. Similar to what was shown in Fig. 1, all cells in the CD4+CD28− T cell line were positive for CD70, whereas CD28+ T cells had low or no CD70 expression. Adding CD4+CD28− T cells to the anti-CD3-stimulated T cells enhanced cell cycle entry and accelerated proliferation (Figs. 5A) as compared with when CD4+CD28− T cells were added (Fig. 5D). In the presence of CD4+CD28− T cells, cocultured T cells had about two more population doublings within the culture period of 5 days. This proliferation-accelerating function of CD4+CD28− T cells was completely inhibited by adding an Ab to CD70 (Fig. 5B), but not by adding a control Ab (Fig. 5C). The phenomenon was not limited to patients with RA, but was also seen with CD4+CD28− T cell lines from healthy donors who were selected to have expanded CD4+CD28− T cell populations (data not shown). This result indicated that the observed enhanced proliferation is genuine to CD70-mediated bystander stimulation.

Although expression of the CD70 receptor CD27 is variable on memory T cells depending on the age and immune status of the donor, CD27 is expressed on all naive T cells (19). We wanted to examine whether CD70-mediated costimulation equally affects naive and memory CD4 T cells. CD4 T cells were separated based on their CD45RA and CD45RO phenotypes and activated with suboptimal concentrations of anti-CD3 and anti-CD28 mAb in the presence or absence of CD28− T cells. Results are shown in Fig. 6. The presence of CD4+CD28− T cells clearly facilitated cell cycle entry and proliferation of naive CD4 T cells in response to suboptimal stimulation. The response again was sensitive to CD70 blocking, which decreased the frequency of cells that entered the cell cycle, although the costimulatory effects of CD28− T cells were only partially reversed. In contrast, no effects on the activation and proliferation of memory CD4 T cells were seen. Neither the addition of CD28− T cells nor the blocking with anti-CD70 mAb significantly influenced the proliferative behavior of CD4 memory T cells.

**CD70 on CD4+CD28− T cells facilitates recruitment of low-avidity naive CD4+ T cells**

The data in Figs. 5 and 6 suggested that CD27 costimulation by CD70 on bystander T cells not only increased the cell cycle progression of stimulated cells, but also facilitated cell cycle entry of naive T cells in response to suboptimal stimulation. To address this question in a more physiological system in which stimulatory and costimulatory molecules are organized in an activation platform between T cells and APCs, we used stimulation with the superantigen TSST in a T cell-DC system. TSST at low concentrations selectively activates Vβ2+ T cells (Fig. 7A). At a concentration of 1 ng of TSST/ml, the Vβ2+ T cell response is maximized. With

**FIGURE 7.** CD70 costimulation favors recruitment of low-avidity CD4 T cells. A. CFSE-labeled naive CD4 T cells were stimulated with autologous DC and increasing concentrations of TSST (0–100 ng/ml, as indicated in the left upper corner of each scatter plot). CFSE dilution was examined by flow cytometry for high (Vβ2+) and low (Vβ2−) avidity T cells. B. CFSE-labeled naive CD4+ T cells were cocultured with both autologous TSST-1-pulsed (1 ng/ml) DC and autologous CD4+CD28− T cells (b and c) or CD4+CD28+ T cells (d). A negative control without TSST-1 is shown in a. To confirm that the costimulatory effect of CD4+CD28− T cells was related to their expression of CD70, cells were preincubated with anti-CD70 mAb (c) or with isotype control mAb (b). CFSE dilutions on Vβ2+ and Vβ2− T cells were examined on day 5.
increasing TSST concentration, more and more $\beta$2-negative T cells are stimulated and enter the cell cycle. We used a concentration of 1 ng/ml TSST to stimulate CFSE-labeled T cells cocultured with autologous DC. In the presence of autologous CD4$^+$ CD28$^-$ T cells, the majority of $\beta$2$^-$, but only very few $\beta$2$^+$, T cells proliferated (Fig. 7B). In contrast, after the addition of CD4$^+$ CD28$^-$ T cells, a large fraction of $\beta$2$^-$ T cells was recruited into the cycle, and the majority of proliferating cells were $\beta$2$^+$. This effect was completely reversible by the addition of an anti-CD70 Ab. These data showed that CD70 expressed on a third-party cell is able to lower the activation threshold to Ag recognition presented by physiological APCs.

Discussion

Our data show an abnormal regulation of CD70 expression in CD4$^+$ T cells that have lost the expression of CD28. The regulatory defect leads to a sustained cell surface expression of CD70 after T cell activation. CD4$^+$CD70$^+$ T cells provide third-party help in the initiation of T cell responses through the CD27-CD70 pathway by lowering the threshold for the activation of low-affinity T cells and, thereby, increasing the risk of autoreactivity. Because CD4$^+$ CD28$^-$ T cell accumulation in RA patients is disproportionate for age, and the frequency of CD70 expression on CD4 T cells in RA is increased, the data provide a model for how CD4$^+$CD28$^-$ T cells could influence RA pathogenesis. The CD27-CD70 receptor-ligand interaction is a well-established pathway of costimulation (16, 18–20). CD27 is expressed on naive CD4 and CD8 T cells, subsets of memory cells, NK cells, and primed B cells (31–34). Costimulatory activity is controlled by the expression of CD70, which is activation dependent and mostly restricted to T cells, DC, and B cells. CD27 stimulation occurs during T cell priming when T cells recognize Ag on CD70-expressing DC or B cells (19). An important role for CD27 stimulation has also been documented for the expansion of effector T cells and the accumulation of CD8$^-$ effector T cells at the site of infection (35, 36). Stimulation of CD27 on primed B cells plays a role in the expansion of centroblasts and, at least in humans, promotion of plasma cell differentiation (37, 38). CD27 signals through TNFR-associated factor-2/5 and NF-kB-inducing kinase, leading to the activation of the NF-kB and c-Jun kinase pathways and promoting cell survival and differentiation (39). Recent experiments in CD27 knockout mice have emphasized the importance of CD27 stimulation for CD8 T cell responses (40). In the absence of CD27 costimulation, the development of CD8 effector and memory T cell responses is impaired; the likely mechanism is that CD27 stimulation inhibits the CD8 T cell contraction that normally occurs after Ag-induced clonal expansion. Studies in transgenic mice that aberrantly express CD70 are of particular relevance for the findings presented in this work. Arens et al. (40) report the induction of protective immune responses against weakly immunogenic tumor cells that were lethal in wild-type mice, consistent with our finding that CD70 costimulation lowers the threshold for T cell activation. Although autoimmunity was not a general finding with our finding that CD70 costimulation lowers the threshold for Ag recognition pre-effect was completely reversible by the addition of an anti-CD70 Ab. In contrast, after the addition of CD4$^+$ CD28$^-$ T cells, a large fraction of $\beta$2$^-$ T cells was recruited into the cycle, and the majority of proliferating cells were $\beta$2$^+$. This effect was completely reversible by the addition of an anti-CD70 Ab. These data showed that CD70 expressed on a third-party cell is able to lower the activation threshold to Ag recognition presented by physiological APCs.

In this context, it is of particular interest that RA patients combine a clinical picture of autoimmunity with evidence of age-inappropriate repertoire contraction and telomere shortening in naive CD4 T cells as signs of premature immune aging (2, 21, 42, 43). The mechanisms underlying these findings and their relationship to developing autoimmunity are unclear. We have hypothesized that RA patients go through a stage of increased turnover, leading to repertoire contraction and selection of an autoimmune repertoire (44). It remains to be examined whether mice that constitutively express CD70 on cells other than APCs develop a phenotype with moderate clonal exhaustion and dominant features of autoimmunity, resembling the picture in RA.

We used a superantigen-driven system with TSST to probe naive CD4 T cell responses in a semiphysiological setting. In this system, CD70 costimulation particularly favored the responses of $\beta$2-negative T cells that are usually only minimally responsive to TSST. Langenkamp et al. (27) have suggested that $\beta$2$^-$ T cells responding to TSST represent low-affinity T cells. The system therefore allows for comparing high-affinity and low-affinity human T cell responses, which is otherwise not possible because of the low frequency of human T cells to nominal peptides. Superantigen-mediated T cell stimulation employs regular T cell recognition synapse formation; however, recent studies have also shown that superantigens can bypass the Lck dependence of the TCR signaling pathways (45), suggesting that superantigen stimulation is an imperfect, although currently the best, experimental system to study human naive T cell responses. In particular, TSST may be the best superantigenic model because of the unique structure of the MHC-TSST-TCR complex (46). With this caveat in mind, data obtained in this system appear to be informative for T cell responses to nominal Ags.

Costimulatory molecules on T cells generally cocluster with the T cell activation complex induced by the recognition of antigenic peptide/MHC and costimulatory ligands expressed on the same APC. Our results show that CD27 stimulation in trans position to the TCR by encountering CD70 on cells other than the APC augments T cell responses. The enhancing effect of third-party CD70 was even observed when T cells were stimulated by DC that expressed CD70 by themselves. Other studies have also shown bystander activity of CD70; application of soluble CD70 in vivo augmented CD8 T cell responses after immunization with the OVA peptide (47). It remains to be seen whether CD27 stimulatory signals in trans and cis positions relative to the TCR differ in their signaling cascades and functional outcomes.

CD70 overexpression is not only seen in RA patients; it has also been described in SLE (29, 48). The mechanisms are different, but the functional consequences appear to be very similar. Lu et al. (28) have proposed that CD70 overexpression in SLE is caused by DNA demethylation of the CD70 promoter between positions −515 and −423. This region was only partially methylated in SLE patients, and demethylation of this region increased CD70 transcription. The mechanism of CD70 overexpression in CD4$^+$ CD28$^-$ cells is different; we have not found any differences in the demethylation patterns between CD4$^+$ CD28$^+$ and CD4$^+$ CD28$^-$ T cells. The proximal CD70 promoter is fully demethylated in resting cells between positions −9 and −167, which is apparently sufficient to allow activation-induced CD70 expression. Mechanisms that down-regulate CD70 expression after activation are obviously important, but remain undefined. Our experiments indicate that whereas DNMT inhibition can increase CD70 expression, activation-induced transcription does not involve DNA demethylation of the CD70 promoter, and DNA demethylation fails to explain why CD28$^-$ T cells have lost this timely ability to revert to CD70-negative cells.
Uncontrolled expression of CD70 could not only induce a chronic T cell response, but also eventually compromise the naive T cell repertoire, as seen in RA. CD70 therefore emerges as a therapeutic target in RA, e.g., by interfering with CD27-CD70 receptor-ligand interaction. Preferably, approaches can be developed to repress CD70 expression. In contrast to SLE, the deficit in RA does not appear to involve epigenetic DNA methylation, suggesting that understanding the activation-induced transcriptional machinery in CD4+CD28- T cells will provide new therapeutic opportunities.

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