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Aberrant Regulation of Synovial T Cell Activation by Soluble Costimulatory Molecules in Rheumatoid Arthritis

Bing Wan,* Hong Nie,* Ailian Liu,* Guozhang Feng,† Dongyi He,† Rong Xu,† Qi Zhang,* Chen Dong,‡ and Jingwu Z. Zhang*§¶

T cell activation and function are critically regulated by positive and negative costimulatory molecules. Aberrant expression and function of costimulatory molecules have been associated with persistent activation of self-reactive T cells in autoimmune diseases such as rheumatoid arthritis (RA). In this study, initial analysis of costimulatory molecules led to the unexpected observation that, in addition to CD80, several negative regulators (e.g., CTLA-4, programmed death-1 (PD-1), and PD ligand-1) were overexpressed in synovial T cells and macrophages derived from RA patients as opposed to controls. The expression of CD80 and PD ligand-1 on monocytes could be induced in vitro by IFN-γ and TNF-α that were produced abundantly in RA-derived synovial fluid (SF). Furthermore, the soluble form of negative costimulatory molecules occurred at high concentrations in sera and SF of RA patients and correlated with titers of rheumatoid factor in RA patients. In particular, the levels of soluble PD-1 were found to correlate significantly with those of TNF-α in SF derived from RA patients. Detailed characterization of soluble PD-1 revealed that it corresponded to an alternative splice variant (PD-1Δex3) and could functionally block the regulatory effect of membrane-bound PD-1 on T cell activation. Our data indicate a novel pathogenic pathway in which overexpression of negative costimulatory molecules to restrict synovial inflammation in RA is overruled by the excessive production of soluble costimulatory molecules. The Journal of Immunology, 2006, 177: 8844–8850.

Costimulatory molecules are members of a growing family of receptors and ligands that play an important role in controlling and regulating immune response (1–3). There are many examples demonstrating the role of costimulatory molecules in various pathological conditions, including autoimmune diseases, tumors, and infectious pathologies (4–8). Among the family of costimulatory molecules comprised of receptors and ligands, some are critical to activation of T cells by providing the second signal to TCR cross-linking whereas others, such as CTLA-4, programmed death-1 (PD-1), and programmed death-ligand 1 (PD-L1), are thought to down-regulate T cell responses (9–11). For example, the engagement of PD-1 by its specific ligands, PD-L1 or PD-L2, was reported to inhibit T and B cell proliferation and cytokine production (12–15). The critical role for PD-1 in immune regulation is highlighted by recent gene disruption studies demonstrating that strain-specific phenotypes, such as PD-1-deficient C57BL/6 mice, develop lupus-like autoimmune proliferative arthritis and glomerulonephritis with IgG3 deposition (16), whereas deficiency of PD-1 in BALB/c mice results in a severe autoimmune dilated cardiomyopathy followed by death due to congestive heart failure (17).

However, although the function and interaction of costimulatory molecules in T cell priming in lymph nodes have gained significant understanding, the mechanism of action as to how costimulatory molecules are exactly involved in the activation and regulation of inflammatory autoimmune T cells in vivo remains elusive. In particular, little is known about the role of costimulatory molecules in many human autoimmune conditions. For example, in rheumatoid arthritis (RA) in which inflammatory pathology in the joints is thought to involve activation and hyperactivity of proinflammatory T cells, it is unclear as to how the peripheral immune system that integrates various regulatory mechanisms and networks fails to regulate persistent synovial T cell activation and inflammation. There are preliminary indications that aberrant function of costimulatory molecules may be associated with persistent T cell activation in the rheumatoid synovium (18, 19). It was reasoned that persistent activation of synovial T cells may be attributable to overexpression of positive regulators, i.e., costimulatory molecules capable of augmenting T cell activation (e.g., CD28, ICOS) in synovial T cells and macrophages, and aberrant expression of those negative regulators (e.g., CTLA-4, PD-1) or functional antagonism of these molecules by soluble factors (20, 21). In this regard, there is some preliminary experimental evidence suggesting that some of the costimulatory receptors and ligands, such as CD28, ICOS, and CD86, are slightly overexpressed on synovial T cells and macrophages (18, 19). Dong et al. reported recently that aberrant T cell activation is associated with autoantibody to B7H1
in RA patients (21). However, the functional consequences associated with the expression levels of some of these costimulatory molecules and their clinical relevance to RA awaits further investigation.

It has been of great interest to understand how negative costimulatory molecules that are known to down-regulate T cell activation function in vivo in the context of persistent activation of self-reactive T cells in the rheumatoid synovium. Current speculation is that the expression of these negative costimulatory molecules in synovial T cells and macrophages is insufficient, a hypothesis based on the known function of these costimulatory molecules and the persistent T cell activation seen in inflamed joints in RA. This study was undertaken initially based on such a hypothesis, to evaluate systemically the expression of costimulatory molecules, both positive and negative regulators, in synovial T cells and macrophages derived from RA and controls. Our initial observation described in this study, however, proved the opposite and revealed overexpression of negative costimulatory molecules in RA-derived synovial T cells and macrophages. The investigation was deepened to define the mechanism responsible for the overexpression of the identified costimulatory molecules and further examine the possibility that overexpressed negative costimulatory molecules are functionally antagonized by their soluble forms. The study provides new evidence supporting the role of proinflammatory cytokines abundantly produced in inflamed joints in the induction of negative costimulatory molecules and that the soluble form of negative costimulatory regulators plays a key role in the aberrant function of costimulatory molecules in RA.

Materials and Methods

Patients and specimens

A total of 95 patients with RA were included in the study. All patients fulfilled the American College of Rheumatology criteria for RA. This group included 71 females and 24 males with mean disease duration of 15.2 ± 12.4 years. The age of the patients was 54.3 ± 15.5 years. The demographic and key clinical information of RA patients is summarized in Table I. Complete sets of paired synovial fluid (SF) and peripheral blood specimens from healthy individuals were obtained from 37 of the 95 patients for paired analyses. Additional sets of SF and paired serum specimens (no cells) derived from the remaining 58 RA patients were used only for analyses of protein concentrations of the soluble form of the indicated costimulatory molecules (PD-1, PD-L1, CTLA-4, CD80, and CD86) or TNF-α or TNF-β or R& D Systems, respectively, at the indicated concentrations. Cells were kept in culture at 37°C in a 5% CO2 atmosphere for 48 h and were then harvested for RNA extraction before real-time PCR analysis (23).

Isolation of the cell subsets and monocytes

Paired PBMCs and SF mononuclear cells (SFMCs) were isolated from patients with RA or controls. Cells were centrifuged in RPMI 1640 medium and subsequently resuspended in cold PBS containing 2.5% FBS at a cell density of 10 million cells/ml CD4+ or CD8+ T cells and CD14+ cells were positively isolated using Ab-coated magnetic beads according to the manufacturer’s instructions (Dynal Biotech). An aliquot of positively isolated cell fractions was detached from beads and analyzed for purity of CD4+ or CD8+ T cells and CD14+ cells by flow cytometry while the remaining cell preparation was subject to RNA extraction and real-time PCR analysis. In all cases, the purity of the selected CD4+ or CD8+ T cells and CD14+ cell preparations was always >97%.

T cell stimulation

Purified T cell preparations derived from SF or peripheral blood of RA patients or controls were cultured at 1 million cells/ml in RPMI 1640 medium containing 10% FBS in the presence or absence of a panel of recombinant human cytokines (i.e., IFN-γ, IL-10, or TNF-α; R&D Systems), respectively, at the indicated concentrations. Cells were kept in culture at 37°C in a 5% CO2 atmosphere for 12 h and were then harvested for isolation of CD4+ T cells and CD14+ monocytes before real-time PCR analysis. An aliquot of the resulting cells treated with respective cytokines at a concentration of 25 ng/ml was analyzed for surface expression by flow cytometry.

RNA extraction and real-time PCR

Total RNA was isolated from cell pellets using a RNeasy Mini Kit (Qiagen). Genomic DNA was removed from total RNA before cDNA synthesis using the RNase-Free DNase Set for DNase digestion during RNA purification (Qiagen). RNA was stored at −80°C. First-strand cDNA synthesis was performed for each RNA sample using a Sensiscript RT Kit (Qiagen). Random hexamers were used to prime cDNA synthesis. mRNA expression of CTLA-4, PD-1, BTLA, CD28, ICOS, CD80, CD86, PD-L1, PD-L2, and ICOS ligand were determined by real-time PCR using SYBR Green Master Mix (Applied Biosystems). Thermocycler conditions included an initial hold at 50°C for 2 min and then 95°C for 10 min, which was followed by a two-step PCR program: 95°C for 15 s and 60°C for 40 cycles. PCR determination of mRNA expression of full-length PD-1 (flPD-1), PD-1 delete exon 3 (PD-1Δex3), PD-1 delete exon 2 (PD-1Δex2), and PD-1 delete exons 2 and 3 (PD-1Δex2,3) transcripts, the two-step PCR program: 95°C for 15 s and 60°C for 40 cycles. PCR determination of mRNA expression of full-length PD-1 (flPD-1), PD-1 delete exon 3 (PD-1Δex3), PD-1 delete exon 2 (PD-1Δex2), and PD-1 delete exons 2 and 3 (PD-1Δex2,3) transcripts, the two-step PCR program repeated for 50 cycles (23). Data were collected and quantitatively analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as folds relative to the expression of GAPDH for each sample. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (Ct), cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions. The amount of gene expression was then calculated as the difference (∆Ct) between the mean Ct value of the sample for the target gene and the mean Ct value of that sample for the endogenous control (GAPDH). Relative expression of PCR products was expressed as 2 −ΔΔCt. The primer sequences were as follows: GAPDH, 5'-GTGAGGTCGTCAAGCC-3' and 5'-TGAGTCATGAGGGGCTC-3'; PD-1L, 5'-CCTTTAGGCCATGAAAATG-3' and 5'-GGTGTGTGGCTGCGTACTG-3'; PD-1, 5'-CCTTGAATCCAGGAGTCTTAC-3' and 5'-CAAGTTCCCATGTCCTGAGT-3'; PD-1Δex3, 5'-CCTTGAATCCAGGAGTCTTAC-3' and 5'-CAAGTTCCCATGTCCTGAGT-3'; PD-1Δex2, 5'-CCTTGAATCCAGGAGTCTTAC-3' and 5'-CAAGTTCCCATGTCCTGAGT-3'; PD-1Δex2,3, 5'-CCTTGAATCCAGGAGTCTTAC-3' and 5'-CAAGTTCCCATGTCCTGAGT-3'.
GTCCACAGAGAAC-3', PD-1Δex2, 5'-GGTCTTAGAGAGAGGGCA-3' and 5'-GACACCAAAGCAGGTTT-3'; and PD-1Δex23, 5'-TTGGTTCTTAGGGACATTAGG-3' and 5'-TCTTCTCGCCTCAGGAAA-3'.

Flow cytometry
A total of 0.5 × 10⁶ cells were resuspended in PBS containing 1% BSA (Irvine Scientific) and 0.1% sodium azide (Sigma-Aldrich). For surface expression of the indicated CD or costimulatory markers, cells were incubated with conjugated Abs (BD Biosciences) at the recommended dilution or isotype controls for 30 min on ice. For intracellular staining of CTLA-4, the cells were permeabilized with PBS containing 0.5% saponin buffer (Sigma-Aldrich). The resulting cells were stained with mouse anti-human CTLA-4 mAb (BD Biosciences) and followed by washing. Stained cells were fixed with 1% paraformaldehyde and analyzed by a FACSARia cytometer (BD Biosciences).

Detection of soluble costimulatory molecules and TNF-α by ELISA
Concentrations of the soluble form of the indicated costimulatory molecules were measured quantitatively in SF and sera using ELISA. ELISA kits for soluble CTLA-4, soluble CD80, and TNF-α were obtained from Bender MedSystems and used according to the manufacturer’s procedures. For soluble PD-1 and soluble PD-L1, microtiter plates precoated with Abs (R&D Systems) to PD-1 (2 μg/ml) or PD-L1 (1.5 μg/ml) were incubated with PBS containing 10% FBS to block nonspecific binding. SF or serum specimens were diluted at 1/2 and added to wells in duplicate, along with PD-1 or PD-L1 fusion proteins (PD-1-Fc or PD-L1-Fc; R&D Systems) as standards. The plates were then incubated for 2 h at room temperature. After washing, mouse mAbs (ebioscience) to human PD-1 (2 μg/ml) or PD-L1 (1 μg/ml) were added and incubated for an additional 2 h at 37°C. Following the addition of conjugated goat anti-mouse IgG (Southern Biotechnology Associates), color reactions were developed using 3,3'-5',5'-tetramethylbenzidine substrate and subsequently stopped with H₂SO₄. OD was read at 450 nm and quantified using an ELISA reader equipped with specialized software (Bio-Rad).

Functional assay with soluble costimulatory molecules
Purified CD4⁺ T cells isolated from the SF of RA patients (3 × 10⁶ cells/well in triplicate) were cocultured with irradiated autologous SFMC (1.5 × 10⁵ cells/well) as a source of accessory cells in wells precoated with Abs (ebioscience) to CD3 (1 μg/ml) in the presence or absence of recombinant human PD-1 fusion protein or PD-L1 (R&D Systems) at the indicated concentrations. Human Ig was used as a control. T cell proliferation was determined by the addition of 1 μCi of [³H]thymidine for the last 16 h of the 3-day culture. The incorporation of [³H]thymidine was measured quantitatively in a MicroBeta TriLux liquid scintillation counter (Wallac).

Statistics
Differences in the expression of genes between the groups were analyzed by the Mann-Whitney U test. A Student’s t test was used to analyze the differences between the groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student’s t test. A value of p < 0.05 was considered statistically significant.

Results
Increased expression of selected costimulatory molecules in rheumatoid synovium
A panel of mononuclear cell specimens was obtained from paired blood and SF of RA (n = 23) and 10 patients with OA and analyzed for the expression of a set of known costimulatory molecules, including CTLA-4, PD-1, BTLA, CD28, ICOS, CD80, CD86, PD-L1, PD-L2, and ICOS ligand, by real-time PCR. Blood specimens derived from 22 healthy volunteers were used as a control. The initial PCR analyses revealed that mostly the members of the indicated CD or costimulatory markers, cells were incubated with conjugated Abs (BD Biosciences) at the recommended dilution or isotype controls for 30 min on ice. For intracellular staining of CTLA-4, the cells were permeabilized with PBS containing 0.5% saponin buffer (Sigma-Aldrich). The resulting cells were stained with mouse anti-human CTLA-4 mAb (BD Biosciences) and followed by washing. Stained cells were fixed with 1% paraformaldehyde and analyzed by a FACSARia cytometer (BD Biosciences).

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CTLA-4 and PD-1 in T cells and CD80 and PD-L1 in CD14+ macrophages prompted us to explain an obvious paradox and mechanism as to how persistent synovial T cell activation could coexist with the increased expression of these negative regulators in rheumatoid synovium and why this mechanism of negative regulation does not function properly in controlling persistent synovial T cell activation and proliferation. Several possibilities were considered. With the availability of specific Abs and recombinant soluble costimulatory molecules, we evaluated the possibility that high levels of soluble costimulatory molecules in the SF of RA might affect the regulatory function of overexpressed costimulatory molecules. To this end, soluble forms of the indicated negative costimulatory molecules were measured in a large panel of RA-derived sera and SF along with the controls. As shown in Fig. 3A, soluble PD-1 (sPD-1) but not soluble CTLA-4 occurred at a significantly high concentration in both RA sera and SF when compared with that in control sera and SF (p < 0.05). Similarly, concentrations of soluble PD-L1 and soluble CD80 were also elevated in the same SF and serum specimens of RA (Fig. 3A). Interestingly, serum concentrations of soluble forms of PD-1, PD-L1, and CD80, but not CTLA-4, correlated significantly with titers of rheumatoid factor in RA patients analyzed (n = 95). The r value indicates the calculated regression coefficient.

Alternative splice variants of soluble PD-1

It was of interest to note that PD-1 has several alternative splice variants in addition to the full-length membrane-bound isoform (23, 26, 27). We examined which splice variant(s) corresponded to the secreted form of PD-1 produced at a high level in sera and the SF of RA patients. As shown in Fig. 5A, among three alternative splice variants that have unaffected open reading frame, the PD-1Δex3 was preferentially expressed in peripheral blood and synovial T cells derived from RA compared with the controls, whereas the other variants were not detected in any of the groups. The PD-1Δex3 variant lacks the membrane-spanning domain of the PD-1 molecule but has an untouched extracellular domain, suggesting that the putative translational product is soluble PD-1. We further addressed whether the PD-1Δex3 variant was associated with RA or whether its increased expression in SF and sera merely reflected the activation state of T cells in RA. For this purpose, we analyzed the expression levels of this alternative splice variant in T cells that were obtained from RA or controls and activated in vitro with anti-CD3/CD28 Abs. The results showed that the activation state did not significantly alter the expression of the PD-1Δex3 variant in T cells as evidenced by the
same pattern of expression between T cell groups and a comparable level of expression between unstimulated and activated T cells (Fig. 5B). In parallel experiments with purified CD4+, CD8+, and CD14+ cell populations derived from RA patients, PD-1Δex3 transcripts were expressed preferentially in CD4+ T cell fractions (data not shown).

Furthermore, we examined whether soluble PD-1 (i.e., the PD-L1Δex3 variant) and soluble PD-L1 could affect T cell activation in vitro. To this end, recombinant fusion proteins corresponding to the extracellular domains (inclusive of the PD-1 ex3 variant) and soluble PD-L1 could affect T cell activation. In this regard, our study demonstrated that the regulatory properties of membrane-bound PD-1 and PD-L1 were altered by the presence of their soluble forms in the experimental system.

Discussion
In this study, we provide new evidence revealing overexpression of negative costimulatory molecules in RA-derived synovial T cells and APC. The conclusion is supported by a series of analyses involving RA and control synovial and blood specimens by both real-time PCR and flow cytometry. The findings described in this study are not entirely expected by our original hypothesis because of an obvious contradiction between overexpression of costimulatory molecules capable of down-regulating T cell response and persistent activation of self-reactive T cells known to exist in rheumatoid synovium. The observation has prompted us to revise our original hypothesis regarding the role of costimulatory molecules in persistent T cell activation in RA and seek a mechanism to explain the findings contradictory to the known properties of these negative regulators. In contrast to our original speculation that insufficient expression of costimulatory molecules in synovial T cells and macrophages may be associated with aberrant T cell function, the immune system seems to react properly to synovial inflammation by selectively up-regulating costimulatory molecules that are able to inhibit T cell activation. In this regard, our study demonstrates that proinflammatory cytokines (i.e., IFN-γ and TNF-α) produced at increased amounts in rheumatoid synovium are, at least in part, responsible for the induction of expression of these costimulatory molecules on both T cells and macrophages. It is conceivable that persistent T cell activation and overly produced proinflammatory cytokines are seen as a danger signal by the immune system that reacts by selectively up-regulating the expression of negative costimulatory molecules. This possible mechanism may have a broader spectrum to include negative regulators of other family or network, such as Foxp3 and Fas/FasL. Consistent with this notion is our recent finding that IFN-γ is able to induce expression of Foxp3 and peripheral conversion of CD4+CD25+ T cells to CD4+ regulatory T cells in both human and mouse experimental systems.
persistent T cell activation in inflamed joints. In this regard, our study provides compelling evidence that functional antagonism of the overexpressed negative costimulatory molecules appears mediated by their soluble forms secreted at high amounts in sera and the SF of RA patients. This conclusion is based on several lines of evidence indicating significantly elevated levels of soluble PD-1 and PD-L1 detected in sera and the SF of RA patients as opposed to controls. It was further evident that both soluble PD-1 and PD-L1, in a form of recombinant fusion protein, are able to effectively block the function of membrane-bound PD-1 and PD-L1 at a concentration range of >20 ng/ml in the in vitro experimental system. It appears that higher concentrations of recombinant fusion proteins are required to affect synovial T cell function in the system than the concentration of PD-1 and PD-L1 seen in RA SF. That is, the predicted extracellular domain of the mature form of PD-1 contains ~147 aa in length (27), whereas the PD-1 fusion protein used in our study is comprised of the extracellular domain of PD-1 (residues 1–167) and the Fc region of human IgG1 (230 aa) via a linker peptide (R&D Systems, catalog 1086-PD). Thus, the significant structural/conformational differences and glycosylation modifications between the naturally produced sPD-1 and the PD-1 fusion protein are likely to account for the relatively low activity of PD-1 fusion protein seen in our study. It may require higher concentrations of the fusion protein(s) to achieve the same activity in T cell function assays. Alternatively, additional local antagonistic mechanism(s) may synergize with soluble PD-1 and PD-L1 in regulation of T cell autoreactivity in the joint. Nonetheless, the increased levels of soluble PD-1 seen in serum and SF specimens of RA patients are of clinical relevance as the PD-1 levels correlated significantly with those of rheumatoid factor and TNF-α in SF, which may serve as an additional biomarker of local inflammatory reaction.

With the knowledge of possible splice variants of PD-1 (23), we provide new evidence that the detected soluble PD-1 corresponds preferentially to a particular alternative splice variant, PD-1Δex3, that contains the full extracellular domain of PD-1. It is confirmed in this study that the PD-1Δex3 splice variant is specifically associated with RA and not related to the T cell activation state because the expression of this variant is markedly increased in T cells derived from RA and because the expression does not differ between activated and resting T cells. One possible explanation is that the aberrant expression of soluble PD-1 (i.e., the PD-1Δex3 splice variant) in RA may be attributable to genetic polymorphism of the PD-1 gene potentially associated with the disease. Further investigation is warranted to address this possibility.

As described in this study, the expressions of both CTLA-4 and PD-1 were significantly elevated in synovial T cells of RA patients. However, they do not seem to function properly to regulate T cell activation. In the case of PD-1 as described in this study, the aberrant function of elevated PD-1 is associated with the presence of its soluble form, sPD-1. In contrast, this is not the case for CTLA-4. Possibilities exist that there are other forms of antagonists potentially present in RA synovium, such as autoantibodies (20) that may compromise the function of CTLA-4. Recently, CTLA-4 fusion protein (Abatacept) was shown in clinical trials to significantly reduce the disease activity and improve physical function in patients with RA (33–36). As CTLA-4 is one of the main players among many other costimulatory molecules in regulating T cell activation, CTLA-4 fusion protein used therapeutically may improve the regulatory function of membrane-bound CTLA-4 by binding to a potential antagonist(s) in the blood or synovium in RA patients.

Taken together, the findings described in this study have important implications in the understanding of the role of proinflammato-


