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The Peptidyl-Prolyl Isomerase Pin1 Regulates Granulocyte-Macrophage Colony-Stimulating Factor mRNA Stability in T Lymphocytes[1]

Stephane Esnault,[2] Zhong-Jian Shen,[2] Emily Whitesel, and James S. Malter[3]

Cytokine production is associated with both the normal and pathologic inflammatory response to injury. Previous studies have shown that the immunosuppressants cyclosporin A or FK506, which interact with the peptidyl-propyl isomerases cyclophilin A and FK506-binding protein (FKBP12), respectively, block cytokine expression. A third member of the peptidyl-propyl isomerase family, Pin1 is expressed by immune and other cells. Pin1 has been implicated in cell cycle progression, is overexpressed in human tumors, and may rescue neurons from τ-associated degeneration. However, the role of Pin1 in the immune system remains largely unknown. In this study, we analyze the role of Pin1 in GM-CSF expression by human PBMC and $CD4^+$ lymphocytes. We show that Pin1 isomerase activity is necessary for activation-dependent, GM-CSF mRNA stabilization, accumulation, and protein secretion, but not non-AU-rich elements containing cytokine mRNAs, including TGF-β and IL-4. Mechanistically, Pin1 mediated the association of the AU-rich element-binding protein, AUF1, with GM-CSF mRNA, which determined the rate of decay by the exosome. ***The Journal of Immunology,*** **2006, 177: 6999–7006.**

Therapy directed at suppressing the immune response is critical for the treatment of allergy and autoimmune diseases as well as the preservation of HLA-incompatible organ transplants. Immunosuppression can be accomplished by depleting cells, inducing tolerance, preventing the production of proinflammatory mediators, or blocking cell surface receptors (1, 2). The predominant immunosuppressive drugs presently in clinical use consist of glucocorticoids and calcineurin inhibitors. The latter group includes cyclosporin A (CsA)[4] and FK506, which bind to cyclophilin A and FK506-binding protein (FKBP12), respectively. The cyclophilin/inhibitor complex interacts with and blocks calcineurin (3), a protein phosphatase essential for NF-AT activation (4). Cyclophilin A and FKBP12 also display intrinsic peptidyl-propyl isomerase (PPIase) activity. These enzymes recognize and catalyze the *cis-trans* isomerization of X-proline peptide bonds (5–8). Phosphorylated Ser or Thr adjacent to proline (pSer/pThr-Pro) cannot be isomerized by cyclophilin A or FKBP12. Instead, this motif is specifically and solely recognized and isomerized by a related PPIase, Pin1 (9, 10).

Pin1 is a bipartite enzyme containing an N-terminal WW domain that binds to the pSer/Thr-Pro motif, and a C-terminal isomerase domain (11, 12). More than 30 Pin1 targets have been identified with isomerization, causing critical changes in their protein-protein interactions, turnover, phosphorylation status, or localization (13–15). Pin1 is itself tightly regulated, with dephosphorylation increasing isomerase activity (16). The initial and most completely described Pin1 targets are cell cycle regulators, including the protein never in mitosis A and cyclin D1, among others (11, 17). Consistent with such a function, overexpression of Pin1 has been observed in many human cancers, and its levels are predictive of cancer recurrence (18, 19). Recently, Pin1 has also been implicated in τ dephosphorylation, which prevents neurodegeneration observed in animal models of Alzheimer's disease (20) and the processing of amyloid precursor protein (21).

In addition to these important functions, we have recently implicated Pin1 in the control of cytokine expression by activated eosinophils (22). Cell activation with hyaluronic acid triggered Erk phosphorylation, ultimately culminating in GM-CSF secretion (23). Therefore, we asked whether Pin1 was involved in the regulation of GM-CSF mRNA accumulation and production by T lymphocytes. GM-CSF is a hemopoietic growth factor, which activates circulating leukocytes, including APCs, T lymphocytes, and eosinophils (24). It is well established that GM-CSF production in T lymphocytes is controlled by posttranscriptional mechanisms through the AU-rich elements (ARE) in its 3′ untranslated region (25, 26). However, the mechanisms controlling GM-CSF mRNA decay remained largely unknown. We now present data that Pin1 isomerase activity is required for the elaboration of GM-CSF by activated T lymphocytes. Mechanistically, Pin1 regulates cytokine decay by interacting with and modulating the mRNA-binding activity of AUF1. These data suggest that Pin1 inhibitors could have value as anti-inflammatory therapeutics.

Materials and Methods

Reagents

Actinomycin D, mannitol, 5,6 dichlorobenzimidazole riboside, PHA, PMA, and juglone were provided by Sigma-Aldrich. Anti-CD3 and anti-CD28 Abs were purchased from R&D Systems. Polyclonal anti-Pin1, anti-tristetraprolin (TTP), and control IgG were purchased from Santa Cruz Biotechnology; polyclonal anti-AUF1 was from Upstate Biotechnology. Monoclonal anti-HuR was from Molecular Probes (clone 19F12); monoclonal anti-PM-Scl 75 was from J. Wilusz (Robert Wood

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[4] Abbreviations used in this paper: CsA, cyclosporin A; FKBP12, FK506-binding protein; ARE, AU-rich element; [pNA], free *p*-nitroanilide; PPIase, peptidyl-propyl isomerase; qPCR, real-time PCR; TTP, tristetraprolin.

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Johnson Medical School, Piscataway, NJ). HRP-conjugated anti-rabbit or anti-mouse (secondary Abs) and the ECL immunoblot detection system (Amersham-Pharmacia) were used to detect primary Abs.

Preparation of PBMC, CD4$^+$ T lymphocytes and CD14$^+$ monocytes, and culture

Peripheral blood was obtained by venipuncture from volunteer healthy donors with appropriate Institutional Review Board approval. Briefly, heparinized whole blood was centrifuged (700 $\times$ *g*, 20 min) over a Percoll density gradient (density 1.090 g/ml; Pharmacia Biotech) to separate mononuclear cells from granulocytes. The mononuclear cells (lymphocytes, monocytes, NK cells) were then washed in RPMI 1640 and cultured in 24-well dishes at 2.5 $\times$ 10^6 cells/ml with RPMI 1640, 10% FBS, and antibiotics. CD4$^+$ and CD14$^+$ cells were separated from PBMC by negative selection using CD4$^+$ T Cell Isolation Kit II and the Monocyte Isolation Kit II from Miltenyi Biotec. PBMC and CD4$^+$ lymphocytes were activated with PHA (5 μg/ml) plus PMA (10 ng/ml) or with anti-CD3 (coated on plates at 10 μg/ml) plus soluble anti-CD28 (5 μg/ml), and treated either with juglone (1 or 0.1 μM) or TAT-WWPin1 (20 nM). The CD14$^+$ cells were activated with IFN-γ (1 ng/ml) plus LPS (10 ng/ml). The WWPin1 cDNA was provided by P. Lu (Harvard University, Boston, MA) and cloned into pTATHA, and the fusion protein was expressed, as described previously (27).

PPIase activity in cell lysates

Pin1 activity was measured according to the methods of Janowski et al. (28) and Hennig et al. (29), with slight modifications as follows. Cell lysates were prepared by five freeze-thaw cycles in a buffer containing 50 mM HEPES and 100 mM NaCl (pH 7.0). Total protein (10 μg) in 10 μl was mixed with 70 μl of the HEPES/NaCl buffer supplemented with 2 mM DTT and 0.04 mg/ml BSA. Then 5 μl of α-chymotrypsin (60 mg/ml in 0.001 N HCl) was added and thoroughly mixed. Finally, 5 μl of the substrate Suc-AEPF-pNa (provided by Peptides International) dissolved in DMSO and prepared at 100 μg/ml in 480 mM LiCl/trifluoroethanol was added. The absorption at 390 nM, which detects the formation of free *p*-nitroanilide ([pNA]), was monitored using a Beckman Coulter DU 800 spectrophotometer. All of the reagents and materials were kept at 4°C during the procedure. The amount of [pNA] was calculated as follows: absorbance (OD)/9620 M^{-1} cm^{-1} (extinction coefficient)/1 cm. The Δ[pNA]/g of protein was the difference between [pNA] at absorbance maximum and [pNA] at t_0 (first OD value) divided by total protein in grams.

Cytokine protein and mRNA determinations

After 5 h of culture, PBMC supernatant was used for ELISA. Total RNA was extracted from treated PBMC cultured for 4 h with Tri-Reagent (Molecular Research Center), as recommended by the manufacturer. GM-CSF was measured with a modified sandwich ELISA, as described previously (30), using solid-phase and biotinylated solution-phase detection Abs (BD Pharmingen). The sensitivity was <3 pg/ml. Average results from three donors ± SD are shown. GM-CSF mRNA was quantified by reverse transcription, real-time PCR (qPCR). The primers for the qPCR were selected using the TaqMan software, and the SYBR green master mix from Applied Biosystems was used as recommended by the manufacturer. PCR was performed on an ABI 7500 thermocycler (Applied Biosystems). For each set of primers, the PCR efficiency was >97% based on serial cDNA dilutions. The $\Delta\Delta$CT method was used to calculate the fold change of target mRNAs from sample to sample. Ribosomal protein S26 mRNA was used as a housekeeping gene.

Immunoprecipitation and immunoblot

After cell culture, cells were frozen at −80°C, and cytoplasmic lysates were prepared, as described previously (31). For immunoprecipitation, 2–5 μg of Ab was added to each sample, followed by incubation with rocking for 2–4 h at 4°C. For experiments requiring RNase treatment, RNase A (10 μg/ml) and RNase T1 (100 U/ml; both from Calbiochem) were added to the lysates immediately after preparation. Protein G-agarose beads (Sigma-Aldrich) were added, and the incubation was continued overnight. Pellets were washed five times with lysis buffer, and at the last wash the beads were either split for Tri-Reagent (Molecular Research Center) for RNA extraction or dissolved in SDS-PAGE loading buffer for immunoblot.

Statistical analysis

The results were expressed as mean ± SD. Statistical analysis was performed by using unpaired Student's *t* tests. Values of $p < 0.05$ were considered statistically significant.

Results

PPIase activity of Pin1 is increased after PBMC activation

Pin1 has been detected in most mammalian cells, but its activity and function in PBMC have not been elucidated. We measured the PPIase activity of Pin1 in cell lysates against a model, *cis*-oriented peptide substrate (Suc-AEPF-pNa). Once isomerized to the *trans* conformation, the peptide is cleaved by α-chymotrypsin to release an A_{390}-detectable [pNA]. Fig. 1 shows that Pin1 activity was increased in PHA plus PMA-activated PBMC compared with resting cells. In both cases, juglone showed dose-dependent inhibition of isomerase activity with partial effects at 0.1 μM and complete inhibition at 1 μM. It remained possible that the observed effects reflected inhibition of another PPIase or enzyme. Therefore, we transduced the WW domain of Pin1 into PBMC. The isolated WW domain functions as a dominant negative by competitively blocking the ability of endogenous Pin1 to bind target proteins (16). To introduce the peptide into cells, the TAT penetratin sequence was fused to the N terminus of the WW domain (22). TAT-containing peptides transduce primary cells such as human eosinophils with near 100% efficiency (32, 33). Thus, PBMC were incubated with 20 nM of either TAT-WWPin1 or the control TAT-GFP and treated with PHA plus PMA. A total of 20 nM TAT-WWPin1 decreased Pin1 activity in activated PBMC as much as 0.1 μM juglone (Fig. 1). Therefore, PPIase activity is low in resting cells and rapidly rises after PBMC activation. As juglone and the WW-Pin1 domain were inhibitory, Pin1 is responsible for the observed isomerase activity.

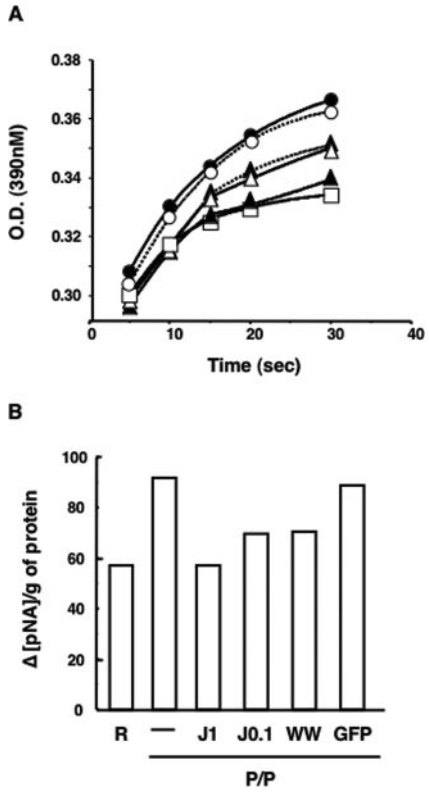

FIGURE 1. PPIase activity is increased after PBMC activation. PBMC were cultured for 3 h without (□, resting (R)) or with PHA plus PMA (●, P/P) in the absence or presence of juglone (1 μM, ▲, J1; or 0.1 μM, Δ, J0.1) or 20 nM TATWWPin1 (....▲...., WW) or 20 nM TATGFP (O, GFP). Cells were washed and lysed before PPIase assay, as described in *Materials and Methods*. *A*, The kinetics of pNA release is representative of multiple experiments. *B*, Δ[pNA]/g of protein was calculated, as described in *Materials and Methods*, and the results are the mean of two experiments.

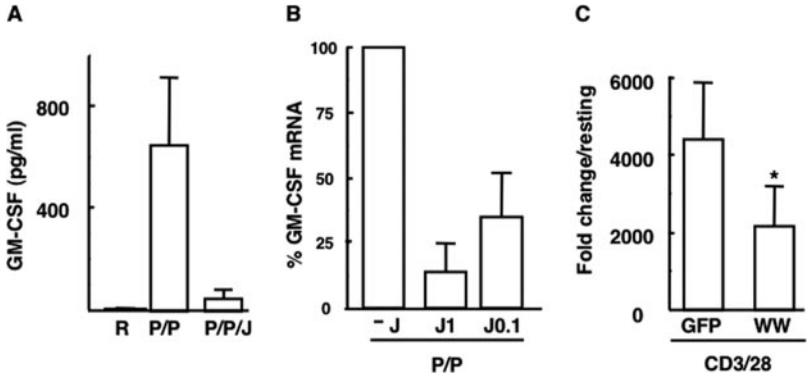

FIGURE 2. Pin1 activity is required for GM-CSF production in activated PBMC. *A*, Resting (R), PHA plus PMA (P/P)-, or P/P plus 1 μM juglone (P/P/J)-treated PBMC were cultured for 5 h. The supernatants were analyzed by ELISA. *B*, GM-CSF mRNA levels were analyzed at 4 h for cells treated as in *A* by qPCR. Results for PHA plus PMA-treated cells (J) have been normalized to 100%, and juglone-treated cells (1 or 0.1 μM) are expressed as a percentage of that value. *C*, PBMC were treated with anti-CD3 plus anti-CD28 with either 20 nM TATWWPin1 (WW) or 20 nM TATGFP as control. GM-CSF mRNA levels were measured by qPCR and presented as fold change compared with resting cells. *, Indicates the value is significantly different ($p < 0.05$) compared with TATGFP treatment. The results (*A–C*) are an average of three different donors ± SD.

Pin1 regulates GM-CSF expression in activated PBMC

Based on prior data with eosinophils (22), we investigated whether Pin1 played a role in proinflammatory cytokine expression by activated immune cells. Therefore, we treated PBMC from healthy donors with PHA/PMA with or without juglone, and assessed GM-CSF cytokine levels by ELISA. At the doses used in this study, juglone has no effect on cyclophilin A or FKBP12 (29). As shown in Fig. 2*A*, 5 h after PMA/PHA, PBMC secreted large amounts of GM-CSF. However, after Pin1 blockade, the secretion of GM-CSF was decreased by ~90% compared with activation-only controls. Analysis after 24 h showed similar results (data not shown). Therefore, Pin1 activity is required for the activation-dependent secretion of GM-CSF by PBMC.

To begin dissecting the mechanism for these effects, we then analyzed GM-CSF mRNA levels by qPCR (Fig. 2, *B* and *C*). As expected after the ELISA results, the level of GM-CSF mRNA was strongly elevated by PHA plus PMA (≈2000-fold, 4 h). When cells were activated in the presence of 0.1 or 1 μM juglone, GM-CSF mRNA accumulation was reduced by >60 or >80%, respectively (Fig. 2*B*). Two housekeeping mRNAs (β-actin and S26 (ribosomal protein)) showed no change after activation with or without juglone (data not shown). Therefore, juglone shows appropriate specificity and appears to selectively prevent GM-CSF mRNA accumulation by activated PBMC. To assess the role of Pin1 in a more physiologically realistic paradigm, PBMC were activated with anti-CD3 plus anti-CD28. GM-CSF mRNA increased by 4000-fold within 4 h. Transduction with TAT-WWPin1 reduced GM-CSF mRNA by ≈50%, whereas TAT-GFP had no effect (Fig. 2*C*). As two mechanistically distinct inhibitors significantly reduced GM-CSF mRNA, we conclude that Pin1 is required for the accumulation of GM-CSF mRNA after immune cell activation.

Pin1 activity is required for GM-CSF mRNA stabilization in activated PBMC

GM-CSF mRNA accumulation after PBMC activation is a balance between mRNA production and decay. Although cytokine mRNAs are typically extremely labile in resting cells, decay is largely attenuated after activation and correlates with protein production (25, 26, 34). Therefore, we investigated whether Pin1 modulates GM-CSF mRNA decay in PBMC. As shown in Fig. 3, GM-CSF mRNA decay was reduced by >3-fold after 4 h of activation with PHA/PMA or anti-CD3 plus anti-CD28 compared with resting PBMC. Pin1 inhibition with juglone or TAT-WWPin1 largely prevented GM-CSF mRNA stabilization after either agonist. The effectiveness in preventing GM-CSF mRNA stabilization (Fig. 3)

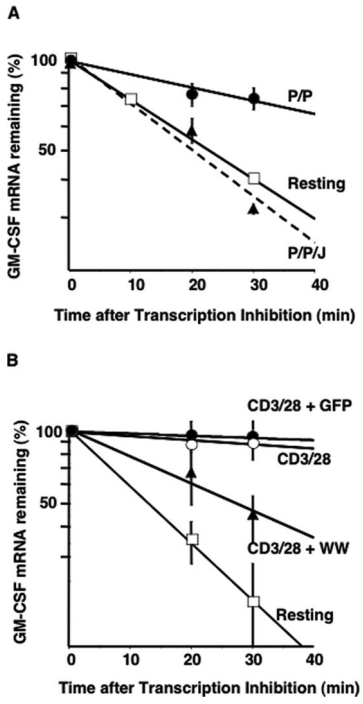

FIGURE 3. Pin1 inhibition accelerates GM-CSF mRNA decay in activated PBMC. *A*, Resting, PHA plus PMA (P/P)-, or PHA plus PMA plus juglone (P/P/J) (1 μM)-treated PBMC were cultured for 4 h. Juglone was added 1 h after the beginning of activation. Actinomycin D (25 μg/ml) was added 4 h after P/P, and the cells were harvested at the indicated time points thereafter. GM-CSF mRNA levels were measured by qPCR. The $\Delta\Delta$Ct method was used, as described in *Materials and Methods*, and mRNA levels at the addition of transcription inhibition were set to 100%. For each point, qPCR was performed in triplicate ± SD. *B*, Resting, anti-CD3 plus anti-CD28 (CD3/28), plus either TAT-WWPin1 (WW) (20 nM)- or the control TAT-GFP (20 nM)-treated PBMC were cultured for 4 h. WW and GFP were added 1 h after the beginning of activation. The 5,6 dichlorobenzimidazole riboside (20 μg/ml) was added 4 h after anti-CD3/28, and the cells were harvested at the indicated time points thereafter. GM-CSF mRNA levels were measured by qPCR. For each point, PCR was performed with three different donors ± SD.

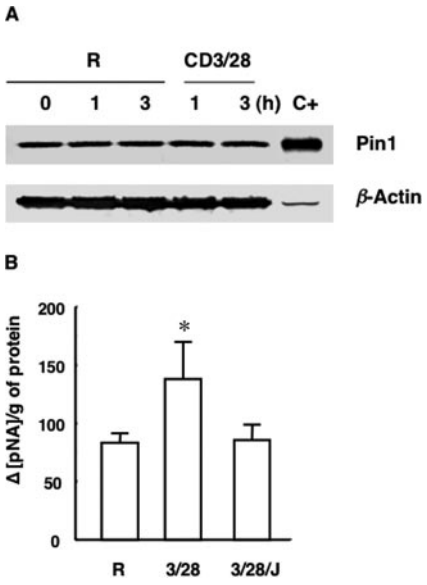

FIGURE 4. Pin1 is activated by mitogenic Abs in $CD4^+$ T cells. *A*, $CD4^+$ T cells were cultured for 1 or 3 h (h) without (R) or with anti-CD3 plus anti-CD28 activation (CD3/28). Pin1 was detected by Western blot with β-actin used as internal loading control. Hela cell lysate was used as a positive control (C^+). *B*, $CD4^+$ T cells were cultured for 3 h without additions (resting (R)) or with anti-CD3 plus anti-CD28 with (3/28/J) or without (3/28) juglone (0.1 μM). PPIase activity was measured in cell lysates, and Δ[pNA]/g protein was calculated, as described in *Materials and Methods*. The results are mean ± SD of three experiments. ∗, Indicates $p < 0.05$ between 3/28- and R- or 3/28/J-treated cells.

correlated well with the inhibitory potency of juglone or the WW domain, respectively (Fig. 1), to suppress Pin1 activity. Therefore, these data strongly suggest that Pin1 regulates GM-CSF mRNA accumulation in PBMC by influencing its decay.

Pin1 activity and function in $CD4^+$ T lymphocytes

The above data implied a role for Pin1 in cytokine elaboration by activated T cells. To verify this, we prepared $CD4^+$ T cells (>98% pure) and analyzed Pin1 protein levels and activity before and after activation. Fig. 4*A* shows that Pin1 was present in $CD4^+$ T lymphocytes, but its amount was unchanged by anti-CD3 plus anti-CD28-mediated activation. However, Pin1 activity was significantly increased after activation, which could be blocked by juglone (Fig. 4*B*). We then investigated the role of Pin1 in GM-CSF elaboration by $CD4^+$ T lymphocytes. Cells were activated with anti-CD3 and anti-CD28, and GM-CSF mRNA was measured by qPCR. Both juglone and the TAT-WWPin1 domain prevented GM-CSF mRNA accumulation (Fig. 5, *A–C*). Of note, even high concentrations of Pin1 inhibitors did not decrease IFN-γ plus LPS-induced GM-CSF mRNA accumulation in $CD14^+$ cells (data not shown). These data suggest a role for Pin1 in cytokine elaboration by activated T cells, but not monocytes.

To assess the breadth of cytokine regulation, we then analyzed the effect of Pin1 inhibition on the accumulation of mRNAs, which show less posttranscriptional control. Unlike GM-CSF, IL-4 mRNA lacks adjacent repeats, but contains two AUUUA elements separated by 3 nt presumably accounting for its greater stability (35). TGF-β mRNA does not possess any AUUUA elements and has a long $t_{1/2}$ (>10 h) in both resting and activated cells (36) (data not shown). As shown in Fig. 5, *A* and *B*, the suppressive effects of Pin1 blockade correlated with the ARE content and susceptibility to decay in resting cells. As for PBMC, Pin1 blockade accelerated GM-CSF mRNA decay in $CD4^+$ T cells (Fig. 6). Therefore, $CD4^+$ T cells require Pin1 activity for GM-CSF elaboration, most likely by modulating the stability of coding mRNA. IL-4 and TGF-β mRNAs were far less affected by juglone than GM-CSF mRNA, suggesting Pin1 regulates only the most labile AU-rich mRNAs.

Pin1 associates with AUF1

AU-rich mRNA-binding proteins have been linked to the control of cytokine mRNA decay. Both stabilizing and destabilizing proteins have been identified (27, 37–41). AUF1 contains a phosphorylated Ser^{83}-Pro^{84}, which regulates AUF1 binding to AU-rich, destabilizing elements (42). This site is a potential Pin1 binding site. Therefore, we immunoprecipitated cytoplasmic extracts with anti-Pin1, followed by Western blotting for AUF1. All four isoforms of AUF1 precipitated together with Pin1 (Fig. 7*A*). Cross-linking followed by immunoprecipitation analysis (CLIP assay) showed in vivo interactions between GM-CSF mRNA and AUF1 in eosinophils (22). Immunoprecipitation results were unchanged after RNase A and T1 treatment (Fig. 7*B*), implicating protein-protein contacts as the foundation of Pin1-AUF1 associations. HuR, another Ser-Pro-containing, ARE-binding protein, was also coimmunoprecipitated with Pin1, but the association was RNA dependent (Fig. 7*B*), suggesting concurrent binding of HuR and AUF1 to GM-CSF mRNA (43). Of note, the ARE mRNA-binding protein TTP was not coimmunoprecipitated with Pin1 (Fig. 7*A*). Despite juglone inhibition, cytoplasmic levels of Pin1 were essentially unchanged in T cells, suggesting Pin1 may undergo differential proteolysis in lymphocytes and eosinophils.

Pin1 regulates AUF1/GM-CSF mRNA interactions in activated PBMC

AUF1 typically functions as a destabilizing protein for AU-rich mRNAs, including c-*myc*, c-*fos*, and GM-CSF (39, 44). Conversely, HuR interactions with mRNAs are thought to protect them from decay (38, 45). Therefore, we analyzed the partitioning of GM-CSF mRNA between AUF1 and HuR in activated PBMC after Pin1 blockade. PBMC were activated with anti-CD3 plus anti-CD28 for 4 h, and juglone (1 μM) was added the last 1 or 2 h of culture. Ten percent of the cell lysate was used to quantify GM-CSF mRNA accumulation (input), and the remainder underwent immunoprecipitation with anti-AUF1 or anti-HuR, followed by RT-PCR for GM-CSF. Pin1 inhibition increased the percentage of GM-CSF mRNA associated with AUF1 and reduced the amount with HuR (Fig. 8*A*). Under these conditions, GM-CSF mRNA decay accelerated (Fig. 6).

AUF1 has been reported to associate with and recruit the exosome, a multiprotein complex with RNase activity that is linked to the rapid decay of ARE-containing mRNA (46). We then analyzed the exosome/GM-CSF mRNA interaction. PM-Scl75, a component of the exosome, was immunoprecipitated, followed by qPCR for GM-CSF. The association of PM-Scl75 with GM-CSF mRNA was then calculated (GM-CSF signal after PM-Scl75 IP/GM-CSF mRNA steady state level). As shown (Fig. 8*B*), Pin1 inhibition for 4 h increased GM-CSF mRNA associated with the exosome by 10,000-fold, which most likely explains the rapid clearance of this mRNA under these conditions.

Discussion

Despite intensive investigation, the signaling cascades leading to mRNA stabilization and subsequent cytokine gene expression remain poorly understood. Progress in this field has been limited by the variability and multiplicity of the signaling pathways activated by different agents in different cell types, and by the complexity

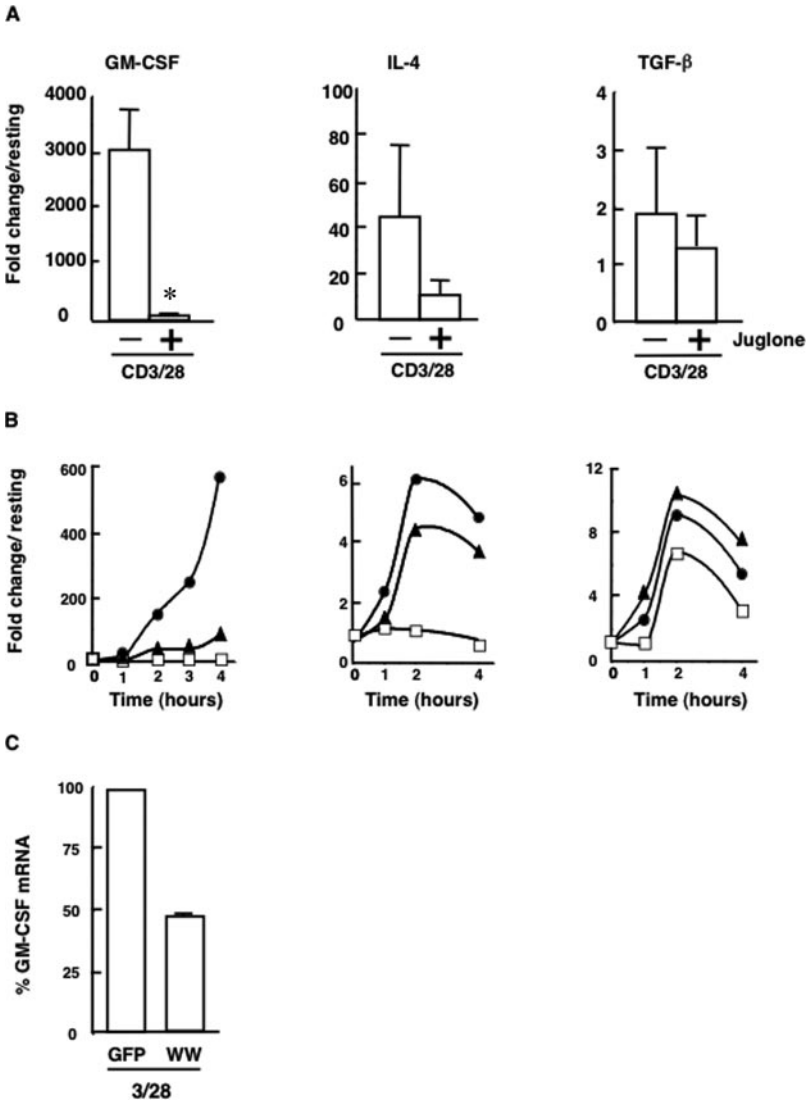

FIGURE 5. Pin1 activity is required for GM-CSF, but not TGF-β or IL-4 mRNA accumulation in activated $CD4^+$ T lymphocytes. $CD4^+$ T cells were not activated or activated with anti-CD3 plus anti-CD28 (CD3/28) for 4 h. *A*, CD3/28-activated cells were untreated (−) or treated (+) with juglone (0.1 μM). GM-CSF, IL-4, and TGF-β mRNA levels were measured by qPCR and presented as fold change from resting cells. The results are mean ± SD of three experiments. ∗, Indicates $p < 0.05$ between 3/28- and juglone-treated cells. *B*, $CD4^+$ cells were untreated (□), or treated with anti-CD3 plus anti-CD28 without (●) or with juglone (▲). GM-CSF, IL-4, and TGF-β mRNA levels were measured by qPCR at the times shown and presented as fold change from resting cells at t_0. *C*, Cells were treated with anti-CD3/anti-CD28 (3/28) and either 20 nM TATWWPin1 (WW) or 20 nM TATGFP (GFP) as control for 4 h. GM-CSF mRNA levels were analyzed by qPCR. Values for GFP were normalized to 100%, and values for WW-treated cells were expressed as a percentage of that value. *C*, Average ± SD of three experiments.

and dynamics of the protein-mRNA interactions thought to be central to this process (47). In this study, we implicate Pin1 in GM-CSF mRNA turnover, accumulation, and cytokine production by human T lymphocytes. In view of the diversity of activators (PHA/PMA and anti-CD3/anti-CD28) and the profound effect on GM-CSF production, Pin1 is most likely a central and essential regulator of GM-CSF mRNA accumulation.

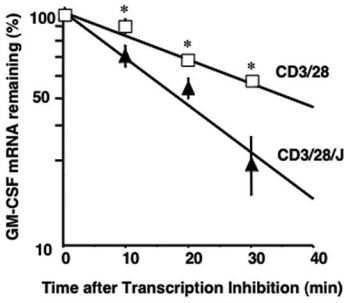

FIGURE 6. Pin1 inhibition accelerates GM-CSF mRNA decay in activated T lymphocytes. Anti-CD3 plus anti-CD28 (CD3/28)- or CD3 CD28 plus juglone (0.1 μM)-treated (CD3/28/J) $CD4^+$ lymphocytes were cultured for 4 h. Juglone was added 1 h after anti-CD3/CD28. Actinomycin D (25 μg/ml) was added 4 h after anti-CD3/28, and the cells were harvested at the indicated time points thereafter. GM-CSF mRNA levels were measured by qPCR using the ΔΔCt method, as described in *Materials and Methods*. Each point is an average ± SD of three experiments. ∗, Indicates $p < 0.05$ between the groups.

We recently demonstrated in eosinophils that Pin1 activity controlled GM-CSF mRNA stability (22). In this study, we provide evidence that Pin1 isomerase activity controls GM-CSF mRNA stabilization in other cells, including human T lymphocytes. Because cytokine secretion depends on and is preceded by mRNA stabilization (25, 26), Pin1-dependent, posttranscriptional regulation most likely accounts for the dramatic suppression of GM-CSF protein secretion observed in this study. This suggests that the production of multiple cytokines, chemokines, and possibly proto-oncogenes, which are highly dependent on variations in mRNA stability, might also be modulated by Pin1 activity. The common features of many of these mRNAs are 3′ untranslated region, AREs. Many RNA-binding proteins have been described that specifically recognize and bind to ARE mRNAs, and regulate their localization, translation, or stability. Among them, the ubiquitous AUF1 and HuR proteins are complementary in function with the former associated with accelerated and the latter, attenuated decay (38, 39, 44, 45). In this study, we show that Pin1 interacts with AUF1 and regulates its function. Pin1/AUF1 interactions were unaffected by cell activation or by Pin1 inhibition. However, modulation of Pin1 activity dramatically changed AUF1 interactions

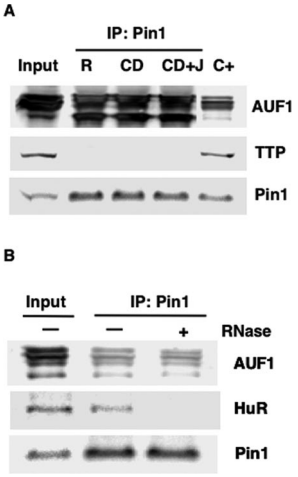

FIGURE 7. Pin1 associates with AUF1. *A*, PBMC were untreated (R) or activated for 4 h with anti-CD3 plus anti-CD28, alone (CD), or with juglone (1 μM) (CD+J) added 1 h after activation. Cell lysates were immunoprecipitated (IP) with anti-Pin1, followed by immunoblot with anti-AUF1, anti-TTP, and anti-Pin1 Abs. Ten percent of total lysate from resting PBMC before immunoprecipitation was used as input. Hela cell lysate was used as a positive control (C^+). *B*, PBMC were activated with anti-CD3 plus anti-CD28 for 4 h. Cell lysates were immunoprecipitated (IP) with anti-Pin1 in presence (+) or absence (−) of RNases, as described in *Materials and Methods*, followed by immunoblot (Abs, *right margin*). Data represent at least three independent experiments with different donors.

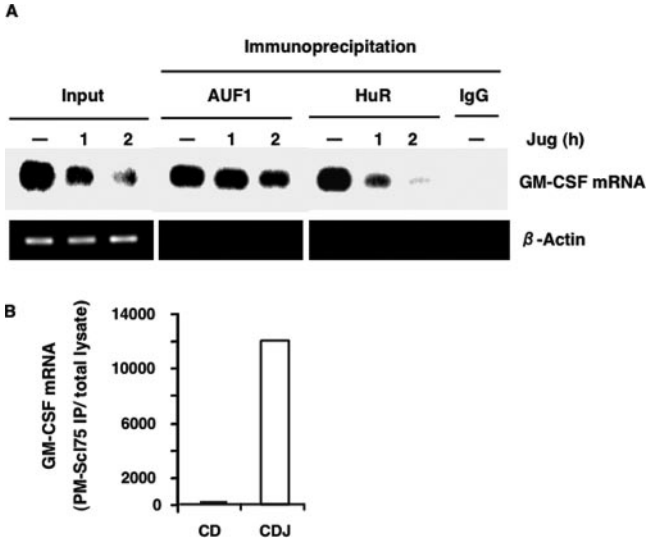

FIGURE 8. AUF1 and exosome interactions with GM-CSF mRNA increase in juglone-treated cells. *A*, PBMC were activated for 4 h with anti-CD3 plus anti-CD28 (—), and juglone (1 μM) was added for the last 1 h (1) or 2 h (2) of the culture. Ten percent of the lysate was used for total RNA isolation (total lysate), and the remaining 90% was used for AUF1, HuR, or nonimmune IgG immunoprecipitation (IP). RT-PCR and Southern blot with radiolabeled GM-CSF cDNA probes were performed on the total lysate and on IP pellets. RT-PCR for β-actin were also performed, and the products were visualized in ethidium bromide gels. *B*, $CD4^+$ cells were activated for 4 h with anti-CD3 plus anti-CD28 alone (CD) or plus juglone (1 μM) (CDJ) added 1 h after activation. RT/qPCR was performed on 10% of the cell lysate (total lysate) or anti-PM-Scl75 IP pellets obtained from the 90% remaining. Data are expressed as a ratio of GM-CSF mRNA associated to PM-Scl75 to GM-CSF mRNA in the total lysate.

with GM-CSF mRNA as well as with the exosome. Therefore, increased Pin1 activity in activated T cells most likely decreases AUF1-binding activity presumably by isomerization, allowing HuR to stabilize GM-CSF mRNA. The decay rate is therefore a function of the relative occupancy of GM-CSF mRNA by HuR and AUF1. Both types of ribonucleoprotein complexes are present in activated cells. This observation could account for the modest stability of GM-CSF mRNA (~30–60 min) in activated cells compared with very stable mRNAs such as globin or actin (>6 h). These data also suggest that other AUF1-regulated mRNAs would show similar Pin1 dependence. In addition, the low or lack of IL-4 or TGF-β regulation by Pin1 compared with GM-CSF reinforces the assumption that Pin1 affects mRNA levels specifically through ARE-binding proteins.

Whereas Pin1 activity has a crucial role in GM-CSF expression by T lymphocytes, monocytes were unaffected by Pin1 blockade (data not shown). The reasons for this observation are unclear, but suggest alternative regulators function in lieu of Pin1 in monocytes to modulate ARE mRNA decay. Consistent with this hypothesis, Pin1 protein levels were much lower in monocytes compared with T cells (data not shown and Fig. 4*A*). Also, different cell types stimulate different intracellular signaling cascades, which can trigger distinct regulation. For example, ERK pathway is triggered in T cells after TCR engagement through *ras* (48), whereas monocytes mainly engage p38 (49). As previously shown, Erk modulates Pin1 activity (22) and GM-CSF production (23), which suggest the intracellular signaling regulating Pin1 in T cells most likely involves Erk.

The possibility that Pin1 controls other steps of mRNA biogenesis in T cells such as transcription cannot be ruled out, but has not been investigated in this study. Juglone at high concentrations (30–100 μM) nonspecifically blocked transcription, altered cell morphology, and accelerated cell death (50). The concentrations used in this study were 30- to 1000-fold lower and had very little effect on cell survival even after 3 days of culture (data not shown). However, Pin1 targets include NF-κB and NF-AT, whose inhibition most likely also contributed to the reduced cytokine mRNA levels seen in this study (15, 51).

As high concentrations of juglone induced cytotoxicity in fibroblasts (52) and Pin1 has been implicated in both apoptosis (53) as well as cell-cycle progression (11, 17), cell death was evaluated. Twenty-four hours after 0.1 or 1 μM juglone, PBMC showed similar levels of survival and apoptosis as untreated controls, as assessed by propidium iodide exclusion and PE-labeled annexin V (data not shown). Therefore, the absence of GM-CSF mRNA and cytokine secretion does not reflect loss of cell viability, implicating Pin1 in activation-induced signaling and posttranscriptional responses.

The lack of total Pin1 blockade with the TAT-WWPin1 shown in Fig. 1 was somewhat expected. Even though TAT-fused proteins or peptides transduce cells with very high efficiency (≈100%) (32), their physical stability as well as activity in cells are highly variable. In our system, TAT-WWPin1 must compete with the endogenous Pin1 for targets to block its activity. Depending on the relative concentrations and affinities for Ser/Thr-Pro motifs, complete inhibition of Pin1 by the WW domain is unlikely. However, juglone irreversibly blocks Pin1 activity by covalently binding to the PPIase active site (29), which is most likely much more effective.

GM-CSF overexpression is associated with significant pathology. Macrophage accumulation with severe tissue damage and inflammatory mediator release (54), autoimmune gastritis (55), lethal myeloproliferative syndrome (56), or eosinophilia (24) all

occur in the context of GM-CSF overexpression. In addition, GM-CSF polymorphisms are highly associated with asthma (57), and patients treated with GM-CSF experience rheumatoid disease secondary to T cell inflammation (58). Therefore, suppression of GM-CSF expression by T lymphocytes during inflammation could be harnessed therapeutically. For example, immunosuppression is induced by CsA and FK506, which bind to cyclophilin A and FKBP12, respectively. These drugs interfere with NF-AT phosphorylation by blocking calcineurin (3), which suppresses GM-CSF, IFN-γ, IL-2, and IL-4 gene transcription. Our data demonstrate Pin1 regulates proinflammatory GM-CSF through a posttranscriptional process involving AUF1. Because Pin1, CsA, and FK506 affect immunomodulator gene expression through distinct mechanisms, combined therapy could be additive or synergistic.

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Disclosures

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