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Interaction between GATA-3 and the Transcriptional Coregulator Pias1 Is Important for the Regulation of Th2 Immune Responses[1]

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Th2 cytokine expression is dependent on the transcription factor GATA-3. However, the molecular interactions of GATA-3 leading to Th2 cytokine gene activation have not been well characterized. Here, we reported a number of GATA-3 associated proteins in Th2 cells, and one of such proteins Pias1 functioned as a positive transcriptional coregulator for GATA-3. When overexpressed in Th2 cells, Pias1 enhanced the expression of IL-13, and to lesser degrees, IL-4 and -5. Conversely, Pias1 siRNA down-regulated the Th2 cytokine expression. In *Leishmania major* infection, manipulating Pias1 expression in parasite-reactive CD4 T cells altered severity of disease caused by Th2 responses. Mechanistically, Pias1 markedly potentiated GATA-3-mediated activation of the IL-13 promoter by facilitating the recruitment of GATA-3 to the promoter. In contrast, IL-5 promoter was modestly enhanced by Pias1 and no effect was observed on IL-4 promoter. Thus, both promoter activation and additional mechanisms are responsible for regulation by Pias1. *The Journal of Immunology,* 2007, 179: 8297–8304.

T he concept of Th1 and Th2 dichotomy is important for understanding the quality and consequences of an immune response (1). While Th2 responses to helminth infection provide protective immunity, inappropriate Th2 responses to allergens and intracellular parasites cause allergic and parasitic diseases such as atopic asthma and leishmaniasis. The Th2 cell effector functions are mediated by their secreted cytokines. Upon TCR stimulation, Th2 cells characteristically produce IL-4, -5, and -13. Both IL-4 and IL-13 can induce IgE class switching (2, 3), and IL-5 induces eosinophil generation and migration (4). Elevated IgE levels and eosinophilia are hallmarks of worm infection and allergic asthma (5, 6). For the protective immunity against worm infection, IL-4 may or may not be required depending on the host genetic backgrounds and the types of worms, whereas IL-13 plays a more dominant role in the resistance to most gastrointestinal nematodes (7–9). IL-13 is also particularly important in allergic asthma for its nonredundant causative role in airway hyperresponsiveness (10, 11). In contrast to the protective role in helminth infection, IL-4 and -13 play a pathogenic role in *Leishmania major* infection, and the two cytokines exert additive effects to promote disease progression (12–14). Thus, among the Th2 cytokines, IL-13 has increasingly been recognized as a critical Th2 effector cytokine in clinical conditions associated with biased Th2 responses.

Given the importance of cytokines in Th2 cell-mediated immune responses, it is necessary to understand how the Th2 cytokine gene expression is regulated. The genes for IL-4, -5, and -13 are closely located on the same chromosome to form the so-called Th2 cytokine gene cluster. It is believed that both global and gene specific mechanisms operate to control the expression of individual Th2 cytokine genes (15). The transcription factor GATA-3 is required for the differentiation of Th2 cells from naive precursors and for the expression of Th2 cytokines in differentiated Th2 cells (16–21). During Th2 cell differentiation, GATA-3 induces active chromatin remodeling and/or histone modification at the Th2 cytokine gene locus that contribute to the global regulation of all three Th2 cytokine genes (22–28). GATA-3 also locally activates the promoters of the Th2 cytokine genes (17, 20, 29–32). In fact, an early study showed that antisense GATA-3 down-regulated the expression of all Th2 cytokines in a Th2 clone (20). More recent studies using conditional knock-out model demonstrated that deletion of GATA-3 in differentiated Th2 cells abolished IL-5 and IL-13 expression and diminished IL-4 expression to minimal levels (19, 21).

Gene regulation at the levels of chromatin remodeling and direct transcriptional activation must be mediated by series of enzymatic reactions. Although the essential role of GATA-3 in Th2 cytokine gene expression has been firmly established, the molecular and/or enzymatic events connecting GATA-3 to cytokine gene activation remain uncharacterized. Further understanding of cytokine gene regulation in Th2 cells would require studies to fill this gap of knowledge. Like many other lineage specifying transcription factors, GATA-3 is not known to possess any intrinsic enzymatic activity. Therefore, GATA-3 must interact with protein partners to form functional complexes to activate cytokine gene expression. So far, 3 proteins Rog, Fog, and Pu.1 have been shown to interact with GATA-3 in CD4 T cells. Although these proteins are important regulators of GATA-3 function, they do not appear to be the partners of GATA-3 for gene activation because all three proteins

function as inhibitors of GATA-3 (33–36). In this paper, we report the identification of a number of GATA-3 associated proteins in Th2 cells. Importantly, we have performed extensive functional studies on one of the identified GATA-3 associated proteins, the transcription regulator Pias1. We found that Pias1 enhanced Th2 cytokine gene expression, in part by potentiating GATA-3 mediated activation of cytokine gene promoters. In the *L. major* infection model, manipulating Pias1 levels in parasite-reactive CD4 T cells altered the severity of the Th2-mediated disease in susceptible mice.

Materials and Methods

Animals and cell culture

DO11.10Ca$^{-/-}$ mice were obtained as gifts from Dr. Daniella Metz and were bred in the University of Rochester Medical Center animal facility. Inbred mice were purchased from National Cancer Institute, National Institutes of Health. All animal studies were performed in compliance to rules stipulated in the policies of the University of Rochester Committee on Animal Research. As previously described (18, 37), for Th1 and Th2 cell differentiation, CD4 T cells were stimulated with OVA$_{223–239}$ peptide (0.5 μg/ml) or ConA (Sigma-Aldrich) (2.5 μg/ml) plus T cell-deleted spleen cells as APC and 50 U/ml human IL-2 (Roche). For Th1 differentiation, the cultures were supplemented with IL-12 (Biogen) (5 ng/ml) and anti-IL-4 Abs (11B11) (BD Pharmingen) (1 μg/ml), whereas for Th2 differentiation, the cultures were supplemented with IL-4 (BD Pharmingen) (10 ng/ml), anti-IFN-γ (XMG1.2) and anti-IL-12 Abs (BD Pharmingen) (1 μg/ml). M12 cells were a gift from Dr. Laurie Glimcher, and 293T cells were purchased from American Type Culture Collection.

Yeast two-hybrid screening

Matchmaker Two-Hybrid System 3 was purchased from Clontech. A fragment of mGATA-3 containing the N-terminal finger (N-f) and C-terminal finger (C-f) and the DNA binding domain was in-frame ligated to the GAL4 DNA-binding domain in plasmid pGBKT7 to produce the GATA-3 bait (BD-GATA-3). Th2 cell cDNA was synthesized using SuperScript Choice System for cDNA synthesis (Invitrogen Life Technologies) and cDNA synthesis module (Amersham Life Sciences). The cDNA library was constructed by ligating the cDNA to the GAL4 activation domain in plasmid pGADT7. Competent AH109 yeast cells were transformed sequentially with the GATA-3 bait and Th2 cDNA library. Transformants were then spread on His-Leu-Trp SD medium plates. Positive transformants were identified and re-streaked on Ade–His-Leu-Trp SD plates containing 5 mM of 3-amino-1,2,4-triazole (3-AT; Sigma-Aldrich) and assayed for β-galactosidase activity. Plasmids from positive clones were purified and cotransferred with the GATA-3 bait or BD-lamin C into AH109 yeast cells to repeat the same screening assays to confirm specific interaction with the GATA-3 bait and not the lamin C bait.

In vitro transcription and translation

BD-GATA3, BD-lamin C and preys including the PIAS1 Gal4 fusion proteins were in vitro transcribed and translated using TNT Quick-coupled transcription/translation system (Promega) according to manufacturer's instructions. Briefly, 1 μg of plasmid DNA templates (pGBKT7-GATA3 or pGBKT-lam or pGADT7-PIAS1) was mixed with 40 μl of TNT Quick Master Mix and with or without 2 μl of [^{35}S]methionine in a total volume of 50 μl and incubated at 30°C for 90 min. Successful synthesis of the proteins was confirmed by SDS-PAGE and Western blot analysis using anti-hemagglutinin (HA)[4] antiserum against the HA-tagged Gal4-activating domain (AD) or with anti-c-*myc* Abs against the c-*myc* tagged Gal4 DNA-binding domain (BD).

Immunoprecipitation

Cells were lysed in high salt lysis buffer (300 mM NaCl, 20 mM Tris-HCl, pH 7.8, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM EDTA, 10 mM ZnSO$_4$) containing proteinase inhibitors. The cell lysates were 1/1 diluted with dilution buffer (lysis buffer without NaCl) and incubated for 1–3 h on ice with Abs recognizing GATA-3 (HG3–31) (Santa Cruz Biotechnology) or HA epitope (Covance) as indicated in each experiments. The protein-Ab complexes were pulled down with protein G beads (Pharmacia) and washed with low salt (150 mM NaCl) lysis buffer. The precipitated proteins

were resolved by 10–12% SDS-PAGE, and analyzed by Western blotting using Abs against GATA-3, HA, or Pias1 (H-175) (Santa Cruz Biotechnology) as indicated.

Retroviral infection of T cells

For overexpression, Pias1 was cloned to the retroviral vector MigR1 (gift of Drs. W. Pears and S. Reiner). The Pias1 sequence gcacggacgcaaacacgaact was used to construct a hairpin siRNA structure expressed under the control of the mouse U6 promoter in the Banshee vector (gift of Drs. G. Hernandez-Hoyos and J. Rossi). BLAST search showed no significant matches of the Pias1 siRNA sequence to other sequence in the mouse genome. Retroviruses were prepared and Th2 cells were infected as described previously (38). Briefly, Pheneox cells (American Type Culture Collection) were transfected with retroviral vectors. Culture supernatants were harvested as viral stock 2 days after transfection. After 24 h of Ag stimulation, T cells were mixed with the viral stocks supplemented with 8 μg/ml polybene and 50 U/ml human IL-2, then centrifuged at 1800 rpm at a tabletop centrifuge at room temperature for 45 min. After overnight incubation, viral supernatants were removed and replaced with the original culture medium.

Analysis of cytokine expression

For intracellular cytokine staining, Th2 cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μM). After 2 h of stimulation Brefeldin A (10 μg/ml) was added to the culture. The cells were incubated for additional 4 h, then fixed and permeabilized using reagents provided in Cytofix/Cytoperm kit (BD Pharmingen). The cells were then incubated with Abs against IL-4, -5, and -13. PE-conjugated anti-IL-4 and anti-IL-5 Abs were purchased from BD Pharmingen. Biotinylated goat anti-mouse IL-13 Abs were purchased from R&D System, and PE-conjugated streptavidin from eBiosciences. For ELISA analysis, CD4 T cells were stimulated with OVA$_{223–239}$ (0.5 μg/ml) plus T cell-depleted spleen cells as APC for 24 h. Culture supernatants were harvested and measured for cytokine concentrations. Capture and biotinylated detection Abs for IL-4 and IL-5 were purchased from BD Pharmingen, and Abs for IL-13 were purchased from R&D System. After incubation with the detection Abs, the sample wells of the ELISA plates were incubated with avidin-conjugated HRP (Vector Laboratories). Color reaction was developed with TMB microwell peroxidase substrate (KPL), and detected at 450 nm wavelength. The statistic significance of cytokine concentrations was determined by the Student *t* test.

L. major *infection*

Metacyclic *L. major* promastigotes (strain WHOM/IR/-/173) were isolated from stationary cultures by negative selection using peanut agglutinin (Sigma-Aldrich). Soluble *Leishmania* Ags were prepared from freeze-thawed promastigotes. Cohorts of BALB/c mice were injected s.c. in the hind leg footpads with 2 × 10^5 metacyclic promastigotes in 50 μl of PBS. Four to 8 wk postinfection, CD4 T cells from draining lymph nodes were isolated by negative selection using magnetic beads (Qiagen). The CD4 T cells were stimulated with soluble *Leishmania* Ags and T cell-depleted BALB/c spleen cells in the presence of 10 ng/ml IL-4. After 1 day of stimulation, the cells were infected with retrovirus. Virus-infected cells were isolated by FACS and i.v. injected into BALB/c Ca$^{-/-}$ mice (5 × 10^5 cells/mouse). BALB/c Ca$^{-/-}$ mice with or without CD4 T cell transfer were infected with L. major on the same day as the cell transfer. Footpad size was measured weekly using a metric caliper and plotted against time postinfection (39). Statistical significance of footpad thickness between experimental groups was determined by the Student *t* test.

Luciferase assay

Sequences for the −800 IL-4, −1.2 kb IL-5 promoters were described previously (17, 31, 32). DNA fragments containing these sequences were amplified from a BAC clone (gift from Drs. R. A. Flavell and G. Lee) by PCR, and cloned to pGL3 luciferase vector (Promega), and verified by sequencing. The IL-13 promoter (−254 to +48) luciferase construct was a gift from Dr. I Ho. Conditions for electroporation were described previously (20). Luciferase activities were measured with the Dual Luciferase System (Promega) according to the manufacturer's instruction using Trilux 1450 Microbeta counter (Wallac). Firefly luciferase activities were normalized to Renilla luciferase activities.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed following a protocol provided by Upstate Biotechnology. Cells were fixed with 1% formaldehyde for 10 min on ice, lyzed and sonicated to yield chromatin fragments. An

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[4] Abbreviations used in this paper: HA, hemagglutinin; AD, activating domain; BD, binding domain.

aliquot of chromatins was saved for determining the chromatin input. The chromatins were immunoprecipitated with anti-GATA-3 Ab (HG3–31) (Santa Cruz) or nonspecific IgG (Upstate Biotechnology). The precipitated chromatins were washed extensively so that no Th2 cytokine gene promoter sequences could be amplified by PCR from chromatin DNA precipitated with the nonspecific IgG. DNA of chromatins precipitated by specific Abs and the input controls were amplified by SYBR green real-time PCR with primers derived from the promoters of IL-4 (aggagagccagtggcaaccta and caatgctggcagaggtctctct), IL-5 (tgtggatcaaagttgtagaaaca and gaatc cccaagcaatttattct) and IL-13 (gcggaagttagctttgctgatg and ccagagccagt gagagaacca) genes using the MyiQ real-time PCR system (Bio-Rad). Amplification of the immunoprecipitated chromatin samples was normalized to the input controls with the $2^{-\Delta Ct}$ method (40). Relative amounts of the normalized amplification were determined among different cell samples.

Results

Yeast two-hybrid screening of GATA-3 associated proteins in Th2 cells

As an effort to characterize transcriptional regulators that partner with GATA-3 to activate cytokine gene expression, we performed yeast two-hybrid screening to identify GATA-3 associated proteins in Th2 cells. We constructed the bait by fusing the GAL4 DNA binding domain with a segment of GATA-3 containing both the N- and C-terminal zinc fingers plus the majority of the sequence between the activation domain and the zinc fingers (Fig. 1A). A cDNA library of differentiated Th2 cells was constructed so that the cDNA fragments were ligated to the GAL4 transactivation domain. Yeast host cells were transfected with the bait and cDNA library. High stringent screening of the library was conducted by performing three different assays of histidine, adenine complementation and lacZ color reaction. The screening identified a number of positive transfectants harboring plasmid clones containing Th2 cell cDNA fragments in-frame fused with the Gal-4 activation domain. Table I summarizes the GATA-3 associated proteins identified in this study. Like GATA-3 itself, most of the GATA-3 as-

sociated proteins are nuclear proteins. The majority of the identified clones contain cDNA derived from three functional groups of genes for transcriptional regulation/sumoylation, RNA processing and cell cycle control (Table I). These results indicate that in addition to gene transcription, GATA-3 may unexpectedly regulate other cellular processes.

Interaction between intact GATA-3 bait and Pias1 prey

We focused further studies on the transcriptional coregulator Pias1. Pias1 is the founding member of the Pias family of transcription factors. Importantly, Pias proteins have been shown to regulate transcriptional activities of GATA family transcription factors including GATA-1, 2 and 4 (41–43). In the yeast two-hybrid screening, Pias1 was one of the repeatedly identified GATA-3 associated proteins. Three clones containing in-frame fused Pias1 cDNA fragments were identified, the longest of which clone G3B886 contained a 2 kb Pias1 cDNA missing only the first 4 amino acids of the coding sequence. Fig. 1B shows the yeast two-hybrid assays of interaction between G3B886 and the GATA-3 bait. When cotransferred with the bait, G3B886 activated the expression of reporter enzymes to support yeast growth in medium lacing histine and/or adenine. It also activated lacZ expression to produce a positive blue color reaction. The interaction between G3B886 and the GATA-3 bait was specific because yeast growth and color reaction were not observed when G3B886 was cotransferred with the lamin C bait.

We further performed coimmunoprecipitation study of in vitro transcribed and translated G3B886 and GATA-3 bait. ^{35}S-labeled G3B886 was incubated with BD-GATA-3 tagged with the c-myc epitope, then immunoprecipitated with anti-c-myc Abs. Electrophoresis and autoradiography of the precipitated proteins showed
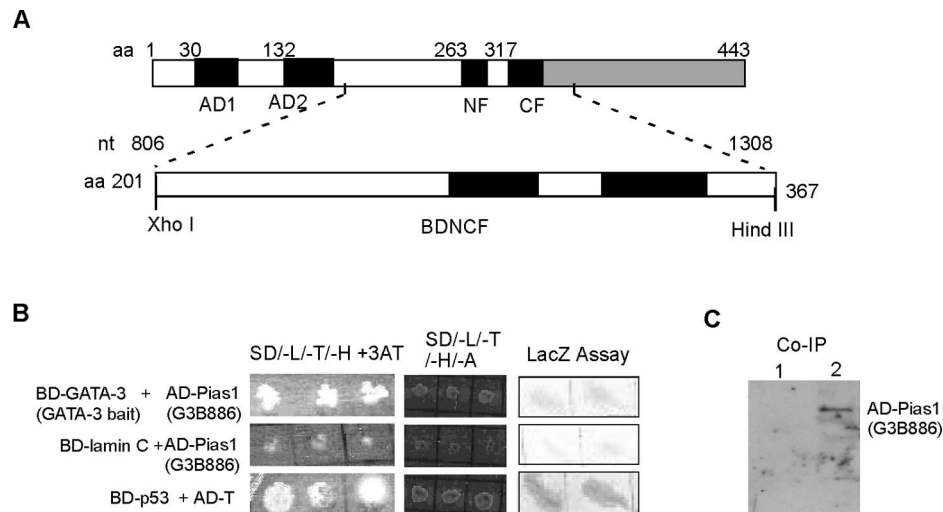
FIGURE 1. Identification of Pias1 as a GATA-3 associated protein by yeast two-hybrid screening. *A*, Structural features of the GATA-3 full-length protein (*top panel*) and the GATA-3 cDNA fragment used to construct the GATA-3 bait (*bottom panel*). NF, N-terminal zinc finger; CF, C-terminal zinc finger. The cDNA fragment in the GATA-3 bait contains both the N- and C-terminal zinc fingers, and the DNA binding domain, and is in-frame fused with the Gal-4 DNA binding domain (BD-GATA-3). *B*, Yeast two-hybrid assays of interaction between GATA-3 bait and Pias1 prey. Yeasts were transformed with GATA-3-BD and the Pias1 clone G3B886 (AD-Pias1). As negative control, yeasts were transformed with the G3B886 and the lamin C fused with the GAL4 DNA binding domain (BD-lamin C). The positive controls were yeasts transformed with p53 fused with GAL4 BD (BD-P53) and SV40 large T fused with GAL4 AD (T-AD). The *left panel* shows the growth of yeast colonies in medium lacking histidine in the presence of 5 mM 3AT. The *middle panel* shows yeast growth in medium lacking both histidine and adenine. The *right panel* shows white-blue assay of LacZ gene activation. *C*, In vitro interaction between BD-GATA-3 (tagged with c-myc epitope) and AD-Pias1 (G3B886). Both proteins, as well as BD-lamin C (tagged with c-myc), were synthesized by in vitro transcription and translation. AD-Pias1 was labeled with [35S]methionine during synthesis. AD-Pias1 was incubated with either BD-lamin C (*lane 1*) or BD-GATA-3 (*lane 2*), and immunoprecipitated with anti c-myc Abs. The precipitated proteins were separated by SDS-PAGE and autoradiographed.

Table I. *GATA-3 associated proteins*

Gene Name	Subcellular Location	Function
Transcriptional Regulation/Sumoylation		
Pias1	Nucleus	Transcriptional coregulator, E3 SUMO ligase
Ubc9	Nucleus	E2 SUMO conjugase
RNA Processing		
hnRNPX	Nucleus	Binds single-stranded nucleotides/RNA
Cpsf3	Nucleus	Pre-mRNA 3'-end formation, poly(A) addition
Cell Cycle Control		
RBBP2	Nucleus	Interacts with retinoblastoma protein
_APC2	N/A	Promotes anaphase progression
Others		
Hsp84	Nucleus/cytoplasm	Heat shock protein, molecular chaperone
Af-9	Nucleus	Associated with acute leukemias
Sec7	Cytoplasm	Promotes guanine-nucleotide exchange on ARF1, 5
p116Rip	Cytoplasm	Targets myosin phosphatase to cytoskeleton

the coprecipitation of the radioactively labeled G3B886. In contrast, G3B886 was not coprecipitated with the lamin C bait (Fig. 1*C*).

Interaction between intact Pias1 and GATA-3

The above studies showed interaction between the truncated Pias1 and the partial structure of GATA-3 in the bait. To investigate interaction between full-length GATA-3 and Pias1, 293T cells were transfected with GATA-3 or Pias1 tagged with HA epitope or both. Cell lysates of the transfected 293T cells were immunoprecipitated with anti-HA Abs. GATA-3 was detected by Western blot in cells cotransfected with both GATA-3 and Pias1, and not in cells transfected with GATA-3 alone (Fig. 2*A*, *left*). In reciprocal immunoprecipitation, Pias1 was coimmunoprecipitated by anti-GATA-3 Abs in double transfected cells and not in cells transfected with Pias1alone (Fig. 2*A*, *right*). We further investigated the interaction between endogenous GATA-3 and Pias1 in Th2 cells. Pias1 was coprecipitated by anti-GATA-3 anitbodies, but not by the nonspecific Abs (Fig. 2*B*).

Retroviral over-expression of Pias1 in Th2 cells enhanced cytokine expression

To determine the functional significance of the interaction between GATA-3 and Pias1, we conducted experiments to over express Pias1 in Th2 cells by retroviral infection. For retrovirus production, the full-length Pias1 cDNA was linked to the GFP gene by an internal ribosomal entry sequence (IRES) in the MigR1 retroviral vector so that Pias1 and GFP were bicistronically expressed in the
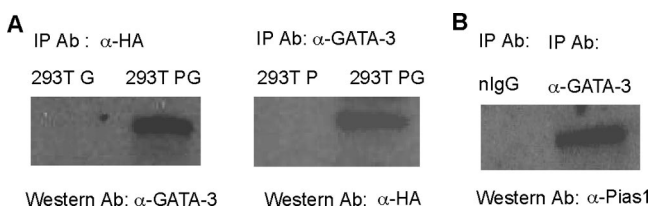

FIGURE 2. Coimmunoprecipitation of full-length GATA-3 and Pias1. *A*, Coimmunoprecipitation with 293T cell lysates. Cell lysates of 293T cells (293T) transfected with GATA-3 or HA epitope-tagged Pias1 alone (293T G, 293T P) or both (293T PG) were immunoprecipitated with the indicated anti-HA or anti-GATA-3 Abs. The precipitates were analyzed by Western blotting with reciprocal Abs. *B*, Coimmunoprecipitation of Th2 cell lysate. Cell lysate of in vitro differentiated Th2 cells derived from DO11.10Ca$^{-/-}$ mice was immunoprecipitated with anti-GATA-3 Abs or nonspecific IgG (nIgG). Western blot of the precipitated proteins was probed with anti-Pias1 Abs.

infected cells. Therefore the virally infected Th2 cells can be identified by GFP expression. CD4 T cells from DO11.10Cα$^{-/-}$ TCR transgenic mice were stimulated for Th2 differentiation for 24 h then infected with retrovirus expressing GFP alone (MigR1) or GFP and Pias1 bicistronically (MigR1 Pias1). After 1 wk of differentiation, infected cells were isolated by FACS based on GFP expression and analyzed for cytokine expression by intracellular staining. Th2 cells retrovirally over expressing Pias1 showed higher percentages of Th2 cytokine-expressing cells, as well as higher levels of cytokine staining (Fig. 3*A*). Among the three Th2 cytokines, the enhancement of IL-13 expression was most dramatic. Albeit to lesser degrees, expression of IL-4 and IL-5 was also enhanced. To further analyze cytokine expression, the isolated CD4 T cells were re-stimulated with OVA$_{223-239}$ peptides plus APC. Cytokine concentrations in the culture supernatants were determined by ELISA (Fig. 3*B*). Consistent with the cytokine staining results, Levels of IL-13 were higher in culture supernatants of CD4 T cells virally expressing Pias1 than those of cells expressing GFP only. Albeit to a much lesser degree, IL-5 production was also significantly increased in cells virally expressing Pias1. In contrast, we did not detect significant difference in IL-4 production. The IL-4 ELISA result was different from that of the cytokine staining, and could be due to different rates of consumption of IL-4 by these two types of cells.

Pias1 siRNA down-regulated cytokine expression in Th2 cells

We next investigated the effects of down-regulating Pias1 on cytokine expression in Th2 cells. We designed a Pias1 siRNA expressed as hairpin structure under the U6 gene promoter in retroviral vector. To test the effectiveness of the siRNA, 293T cells were cotransfected with Pias1 together with the Pias1 siRNA or a nonspecific siRNA construct. Western blot analysis showed that the Pias1 siRNA almost completely inhibited the expression of Pias1 in cotransfected 293T cells (Fig. 4*A*, *left*). We then infected DO11.10Cα$^{-/-}$ Th2 cells with siRNA retroviruses. Like in 293T cells, Pias1 siRNA effectively down-regulated endogenous Pias1 expression in Th2 cells (Fig. 4*A*, *right*). The infected Th2 cells were further analyzed for Th2 cytokine expression by intracellular cytokine staining (Fig. 4*B*). As expected, strong inhibition of IL-13 expression by Pias1 siRNA was observed as judged by the decrease of both the percentages of cytokine expressing cells and the intensity of cytokine staining. To lesser degrees, IL-4 and IL-5 expression was also down-regulated by the Pias1 siRNA. CD4 T cells expressing either control or Pias1 siRNA were also isolated by FACS, and re-stimulated with OVA$_{223-239}$ plus APC to determine cytokine secretion (Fig. 4*C*). As expected, IL-13 production
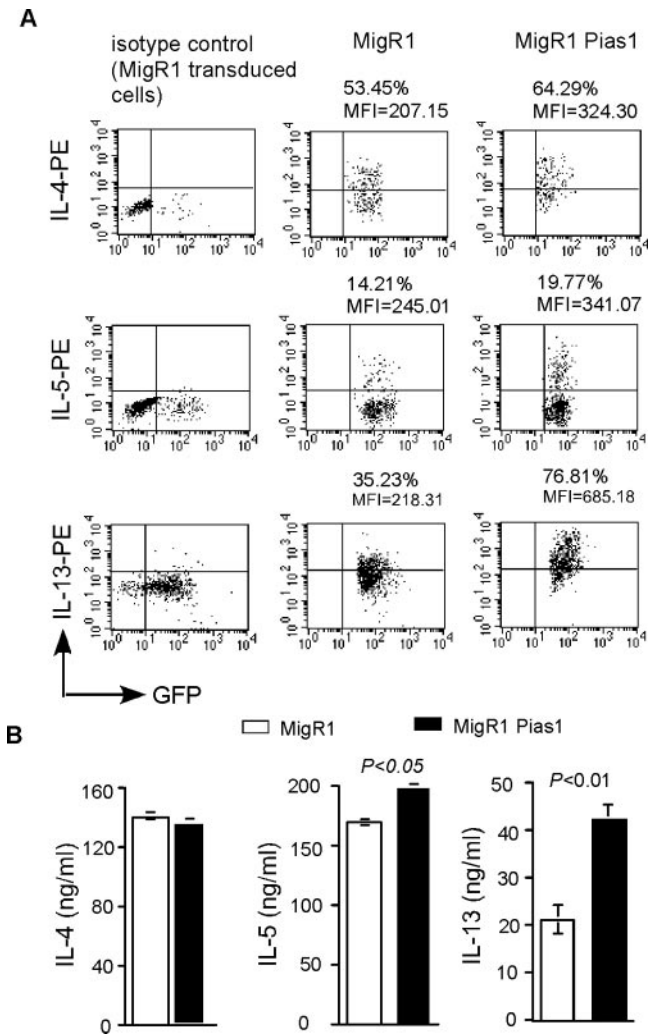
FIGURE 3. Retroviral overexpression of Pias1 up-regulated Th2 cytokine expression. DO11.10Ca$^{-/-}$ spleen cells were stimulated with OVA$_{223-239}$ peptide under Th2 polarizing conditions. The cells were infected with retrovirus bicistronically expressing Pias1 and GFP (MigR1 Pias1) or GFP only (MigR1). *A*, The infected GFP$^+$ CD4 T cells were isolated by FACS and analyzed by intracellular staining of IL-4, IL-5, and IL-13. Dot plots of GFP$^+$ CD4 T cells are shown. Numbers above the plots show the percentages of GFP$^+$ CD4 T cells that expressed the indicated cytokines and the mean fluorescence intensities of cytokine staining of the GFP, cytokine double positive populations. Plots of the three cytokines are derived from different experiments. Representatives of >3 independent experiments are shown. *B*, The virally infected T cells were isolated by FACS and restimulated with OVA$_{223-239}$ and APC. Culture supernatants were harvested and measured for the production of the indicated cytokines by ELISA. Mean values of triplicates of a representative of more than three experiments are shown. Error bars indicate SDs. *p* values were determined by the Student *t* test.

was greatly decreased in cells expressing Pias1 siRNA. IL-5 production was also significantly decreased in these cells, whereas only marginal reduction was observed in IL-4 production.

Effects of Pias1 on L. major *infection*

We used the *L. major* infection model to study the effects of Pias1 on Th2 response in vivo. BALB/c mice infected with *L. major* in the footpad develop pathogenic Th2 responses and footpad swelling. CD4 T cells from draining lymph nodes of infected BALB/c mice were isolated and stimulated in vitro with soluble *L. major* Ags in the presence of IL-4. The restim-

ulated cells were infected with retroviruses expressing GFP alone, or bicistronically with Pias1, the nonspecific siRNA or Pias1 siRNA. The infected cells were adoptively transferred to BALB/c Ca$^{-/-}$ mice. The receipient mice were then infected with *L. major* in the footpads. As shown in Fig. 5, footpad swelling in mice that received CD4 T cells over expressing Pias1 was more severe than mice received CD4 T cells expressing GFP alone. Conversely, footpad swelling was less severe in mice received CD4 T cells expressing Pias1 siRNA than mice received CD4 T cells expressing the nonspecific siRNA. We did not detect significant IFN-γ producing cells in the adoptively transferred CD4 T cells (data not shown). Therefore, the decreased lesion size indicated less efficiency of the transferred cells to induce inflammation rather than homeostatic expansion of residual IFN-γ-producing cells. It should be mentioned that we unexpectedly noticed that over time mice without T cell transfer developed more severe footpad swelling than mice with T cell transfer, suggesting some immunity might have developed even in the nonhealing BALB/c mice. Similar observations were reported in some early studies (44, 45).

Pias1 enhanced GATA-3 mediated activation of cytokine gene promoter

To gain insight into the mechanism by which Pias1 regulates Th2 cytokine gene expression, we studied the effect of Pias1 on the activation of Th2 cytokine gene promoters by luciferase reporter assay. M12 cells were transiently transfected with firefly luciferase reporter gene driven by each of the IL-4, 5 or 13 gene promoters, with or without cotransfection with GATA-3 and/or Pias1. As shown in Fig. 6, Pias1 alone did not activate any of the Th2 cytokine gene promoters. However, when contransferred with GATA-3, Pias1 more than doubled GATA-3-mediated activation of the IL-13 promoter. A modest enhancement of GATA-3-mediated activation of IL-5 promoter was also observed. In contrast, GATA-3 mediated activation of IL-4 promoter was not enhanced by Pias1. Thus, the three Th2 cytokine gene promoters showed different responsiveness to Pias1. Similar results were observed when 293T or Jurkat cells were used in the study (data not shown).

Pias1 facilitated the binding of GATA-3 to endogenous IL-13 promoter

Pias1 has recently been shown to act as transcriptional coregulator by modifying the DNA binding activity of its associated transcription factor (46). Therefore, we predicted that Pias1 might enhance binding of GATA-3 to the Th2 cytokine gene promoters. To test this possibility, we performed chromatin immuoprecipitation (ChIP) experiments to determine whether the recruitment of GATA-3 to the Th2 cytokine gene promoters could be affected by Pias1. Chromatin fragments were prepared from DO11.10.Ca$^{-/-}$ Th2 cells infected with retrovirus expressing GFP alone or together with Pias1 or Pias1 siRNA. The chromatins were immunoprecipitated with anti-GATA-3 Abs. DNA of the precipitated chromatins were analyzed by real-time PCR using primers that amplify DNA sequences containing the GATA-3 binding sites in the Th2 cytokine gene promoters. We found that more IL-13 promoter DNA was precipitated by anti-GATA-3 Abs from cells over expressing Pias1 than cells expressing GFP only, whereas less IL-13 promoter DNA was precipitated from cells expressing the Pias1 siRNA (over 40% reduction) (Fig. 7). In contrast, precipitation of DNA of the IL-4 and IL-5 gene promoters was little affected by Pias1 over-expression or siRNA, suggesting alternative mechanisms may exist.
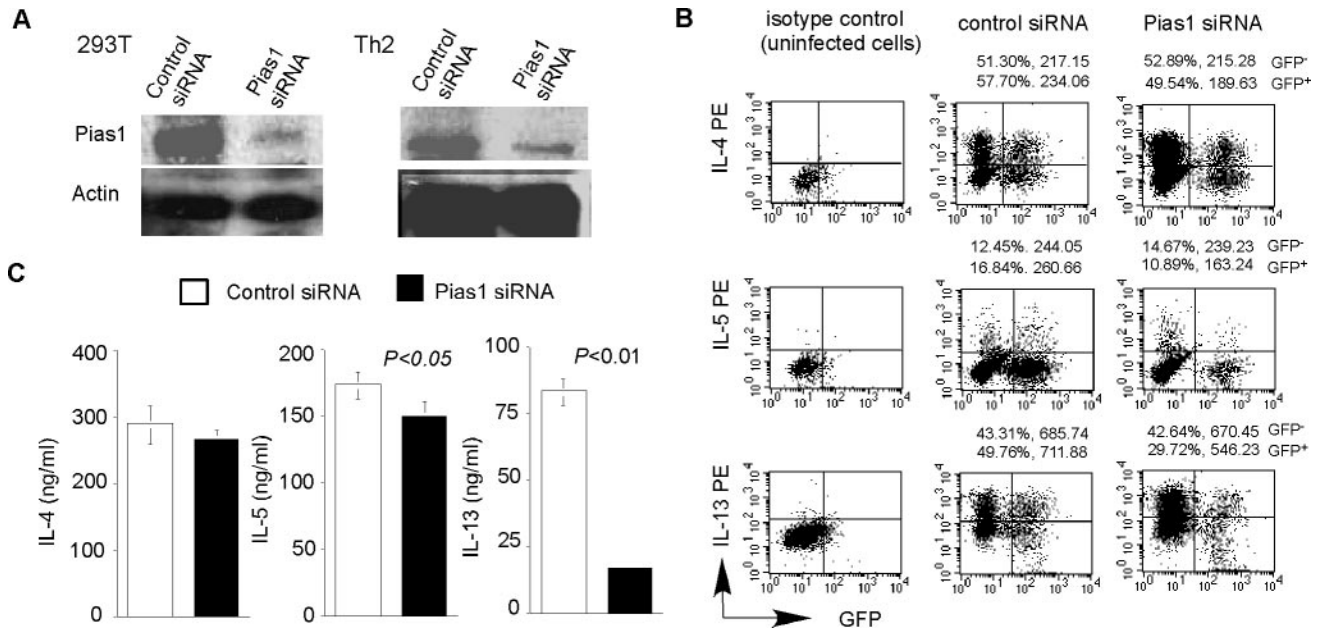
FIGURE 4. Down-regulation of Th2 cytokine expression by Pias1 siRNA. *A*, Inhibition of Pias1 expression by siRNA. In the *left panel*, 293T cells were transfected with Pias1 plus either a nonspecific siRNA or Pias1 siRNA. Cell lysates were analyzed by Western blotting with anti-Pias1 or anti-actin Abs. In the *right panel*, DO11.10C$\alpha^{-/-}$ Th2 cells were infected with retrovirus bicistronically expressing GFP and the Pias1 siRNA or the nonspecific siRNA. The infected cells were isolated and analyzed by Western blotting. *B*, As in Fig. 3, Th2 cells were derived from DO11.10Ca$^{-/-}$ mice and infected with retrovirus bicistronically expressing GFP and the Pias1 siRNA or the nonspecific siRNA. The infected Th2 cells were analyzed by intracellular staining of Th2 cytokines. Numbers above the plots show the percentages of GFP$^-$ or GFP$^+$CD4 T cells that expressed the indicated cytokines, and the mean fluorescence intensities of the cytokine positive populations. The isotype controls were uninfected Th2 cells stained with nonspecific IgG instead of Abs against the cytokines. Representatives of more than three independent experiments are shown. *C*, T cells retrovirally expressing the control or Pias1 siRNA were isolated by FACS and restimulated with OVA$_{223-239}$ and APC. Culture supernatants were harvested and measured for the production of the indicated cytokines by ELISA. Mean values of triplicates of a representative of more than three experiments are shown. Error bars indicate SDs. *p* values were determined by the Student *t* test.

Discussion

Given the importance of Th2 responses in host defense against infection and in disease pathogenesis, the differentiation and cytokine gene regulation of Th2 cells have been under intensive study. Earlier work has found GATA-3 to be the central transcriptional regulator of Th2 cells. However, it remains poorly understood how GATA-3 activates cytokine gene expression. Further understanding of the regulatory mechanism in Th2 cells requires characterization of molecular interactions associated with GATA-3. Toward to this end, we have conducted studies to identify proteins partners of GATA-3 by yeast two-hybrid screening of a Th2 cDNA library. Most of the Th2 cDNA clones identified by this method were derived from genes of three functional clusters of transcriptional regulation, RNA processing and cell cycle control. The identification of genes other than transcriptional regulators suggests that GATA-3 may play unexpected roles in other cellular processes.

Our further studies focused on the transcriptional coregulator Pias1. The results showed that over-expression of Pias1 enhanced, and conversely down-regulation of endogenous Pias1 diminished, Th2 cytokine gene expression. Among the three Th2 cytokines, the expression of IL-13 was most affected by Pias1. IL-13 is a key effector cytokine in Th2 cell-mediated protective immunity and pathogenesis of parasitic and allergic diseases. Therefore, our finding is particularly helpful for understanding the underlying immunopathophysiology in these clinical conditions. It may also suggest that Pias1 could be a potential target for intervening Th2 cell-mediated disease process. Indeed, our study of the *L. major* infection showed that over-expression and down-regulation of Pias1 in parasite-reactive CD4 T cells could increase and decrease lesion

sizes, respectively. Besides IL-13, expression of endogenous IL-4 and IL-5 genes in Th2 cells was also regulated by Pias1 but to much lesser degrees. As discussed below the differences may be partly due to lower sensitivities of the IL-4 and IL-5 gene promoters to Pias1 than the IL-13 promoter. Based on the fact that Pias1 mostly affect IL-13 expression, we believe that Pias1 is a part of the mechanisms that regulate individual cytokine expression in Th2 cells, other than those controlling the entire differentiation program.

GATA-3 functions primarily as a transcriptional activator of Th2 cytokine genes, therefore characterizing positive transcriptional coregulators of GATA-3 in Th2 cells is important for understanding the mode of GATA-3 action. Pias1 is the first GATA-3 coactivator that has been identified. The GATA family transcription factors regulate cell-lineage differentiation and are known to interact with cofactors. GATA-3 is the only GATA factor expressed in T cells. Earlier studies of protein interactions have focused on other GATA factors outside the lymphoid lineages. More recently, several reports have described three proteins Rog, Fog and Pu.1 that can interact with GATA-3 in CD4 T cells (33–36). However, all three proteins appear to be inhibitors of GATA-3 transcriptional activity. Rog was originally described as an inhibitor of GATA-3, but was later found to control T cell activation threshold rather than balance of Th1/Th2 responses under physiological condition (47). Fog is expressed in naive CD4 T cells but is extinguished upon induction of Th1 and Th2 differentiation (34, 36). Therefore, Fog is believed to prevent GATA-3 autoactivation and aberrant Th2 cell differentiation, and may not modulate GATA-3 function in differentiated Th2 cells. In contrast, PU.1 is preferentially expressed in Th2 cells. It suppresses Th2 cytokine
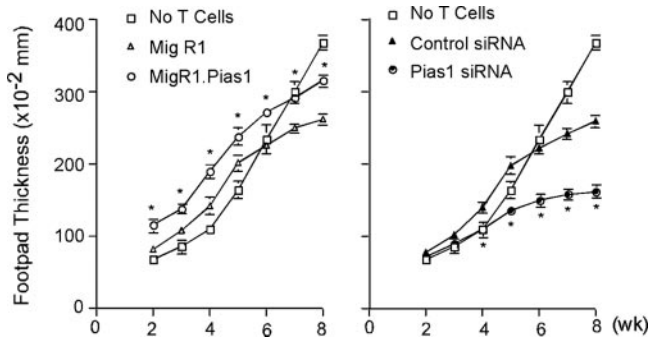
FIGURE 5. Effect of Pias1 on *L. major* infection. CD4 T cells from draining lymph nodes of BALB/c mice infected with *L. major* were isolated and restimulated in vitro with soluble *Leishmania* Ags and APC plus IL-4. The stimulated cells were infected with GFP bicistronic retrovirus expressing Pias1, nonspecific siRNA or Pias1 siRNA. The infected cells were sorted and adoptively transferred to BALB/c Ca$^{-/-}$ mice. The recipient mice were infected with *L. major* and footpad thickness of the mice was recorded. Mean values of four mice for each experimental condition in a representative of three experiments are shown. Error bars indicate SDs. *, Statistically significant differences in footpad thickness between MigR1 and MigR1 Pias1 (*right*), or control and Pias1 siRNA groups (*right*) at the indicated time points ($p < 0.05$).

FIGURE 7. ChIP analysis of the binding of GATA-3 to Th2 cytokine gene promoters. Chromatin fragments were prepared from DO11.10Ca$^{-/-}$ Th2 cells infected with retrovirus expressing GFP alone, together with Pias1 or Pias1 siRNA. The chromatins were immunoprecipitated with anti-GATA-3 Abs. DNA derived from the precipitated chromatins and the input chromatin DNA were analyzed by real-time PCR using primers encompassing the GATA-3 binding sites of the IL-4, -5, and -13 promoters. The amplifications were normalized to the input controls. Mean relative values of triplicates to those of the GFP samples in a representative of three experiments are shown. Error bars indicate SDs. The *p* value of the Student *t* test shows significant difference between Th2. Pias1 siRNA and Th2.GFP.

gene expression by decreasing the binding affinity of GATA-3 to DNA (33). Thus, one may speculate that the balance of the opposing activities of Pias1 and PU.1 may determine the output of Th2 cytokine gene transcription mediated by GATA-3.

As for the mechanism by which Pias1 modulates GATA-3 functions, we found that Pias1 enhanced GATA-3-mediated activation of the IL-13 promoter by facilitating the recruitment of GATA-3 to the promoter. In contrast, although the expression of endogenous IL-4 and IL-5 genes was clearly regulated by Pias1, activation of IL-4 promoter by GATA-3 was not enhanced by Pias1, and only weak enhancement was observed for the IL-5 promoter. Therefore, there are clear differences in the responsiveness of the three cyto-
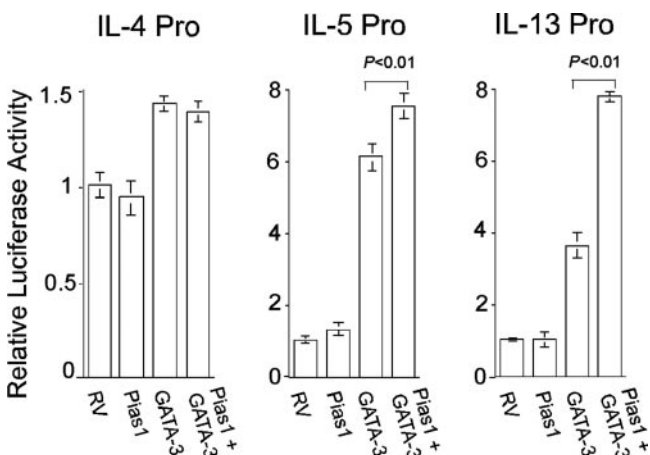
kine gene promoters to Pias1. Logically, such differences must be determined by DNA sequences of the promoters. It has been reported before that Pias1 can modulate the affinity and specificity of the DNA binding activity of its associated transcription factors (46). Furthermore, Pias1 itself can bind to DNA through a SAP domain located at the N terminus (48). Therefore, it is possible that the unique DNA sequences around GATA-3 binding sites may allow Pias1 to bind to DNA. Through such DNA binding and interaction with GATA-3, Pias1 can enhance GATA-3 binding to the IL-13 promoter. Our data of ChIP analysis of GATA-3 binding to the cytokine gene promoters supported this hypothesis. In addition to modulating DNA binding, Pias1 can function as an E3 SUMO ligase in the protein sumoylation pathway. In this regard, it is noteworthy that our yeast two-hybrid screening has also identified Ubc9 (Table I), the only E2 SUMO conjugating enzyme in mammalian cells (49). It is therefore reasonable to assume that GATA-3 may nucleate the formation of a protein sumoylation complex in Th2 cells, in which GATA-3 and potentially other unidentified proteins may be sumoylated. This could be an additional mechanism by which Pias1 regulates Th2 cytokine gene expression.

Disclosures

The authors have no financial conflict of interest.

FIGURE 6. Effects of Pias1 on GATA-3-mediated activation of Th2 cytokine gene promoters. M12 cells were cotransfected with firefly luciferase reporter gene driven by the indicated cytokine gene promoters and Pias1, GATA-3, or empty vector (RV). A construct of Renilla luciferase gene driven by HSV-tk promoter was cotransfected as internal control. The transfected cells were treated with PMA and ionomycin for 24 h, and harvested for luciferase assay. Firefly luciferase activities of the indicated experimental conditions were normalized to Renilla luciferase activities, and their mean values of triplicates relative to the empty vector control in a representative of more than three experiments are shown. Error bars indicate SDs.
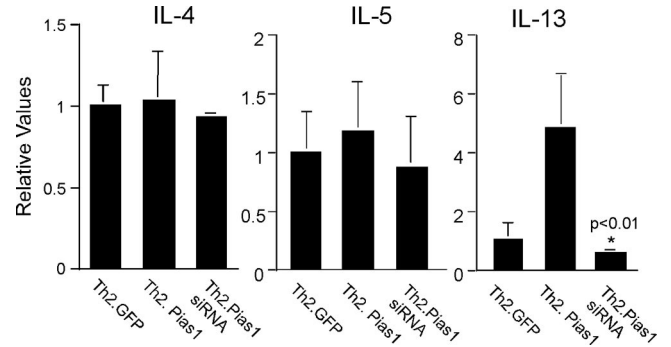
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