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*J Immunol* 2009; 183:4887-4894; doi: 10.4049/jimmunol.0900363
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PU.1 Regulates TCR Expression by Modulating GATA-3 Activity

Hua-Chen Chang,* Ling Han,* Rukhsana Jabeen,* Sebastian Carotta,† Stephen L. Nutt,‡ and Mark H. Kaplan2*†

The Ets transcription factor PU.1 is a master regulator for the development of multiple lineages during hematopoiesis. The expression pattern of PU.1 is dynamically regulated during early T lineage development in the thymus. We previously revealed that PU.1 delineates heterogeneity of effector Th2 populations. In this study, we further define the function of PU.1 on the Th2 phenotype using mice that specifically lack PU.1 in T cells using an lck-Cre transgene with a conditional Sfpi1 allele (Sfpi1lck+/−). Although deletion of PU.1 by the lck-Cre transgene does not affect T cell development, Sfpi1lck+/− T cells have a lower activation threshold than wild-type T cells. When TCR engagement is limiting, Sfpi1lck+/− T cells cultured in Th2 polarizing conditions secrete higher levels of Th2 cytokines and have greater cytokine homogeneity than wild-type cells. We show that PU.1 modulates the levels of TCR expression in CD4+ T cells by regulating the DNA-binding activity of GATA-3 and limiting GATA-3 regulation of TCR gene expression. GATA-3-dependent regulation of TCR expression is also observed in Th1 and Th2 cells. In CD4+ T cells, PU.1 expression segregates into subpopulations of cells that have lower levels of surface TCR, suggesting that PU.1 contributes to the heterogeneity of TCR expression. Thus, we have identified a mechanism whereby increased GATA-3 function in the absence of the antagonizing activity of PU.1 leads to increased TCR expression, a reduced activation threshold, and increased homogeneity in Th2 populations. The Journal of Immunology, 2009, 183: 4887–4894.

The helper cells have the potential to be programmed and differentiated into various subsets of Th cells that are distinguished by their secreted cytokines. The development of the Th2 subset is promoted by the IL-4-STAT6 signaling pathway and the induction of GATA-3 (1, 2). GATA-3 is a transcription factor expressed in T cells that is selectively induced during Th2 development, whereas expression is reduced in Th1 cells (3). GATA-3 functions both by directly binding to Th2 cytokine loci and chromatin remodeling of Th2 cytokine loci allowing other factors to bind more effectively (4, 5). The central role of GATA-3 has been demonstrated using cells and mice with a conditional deletion of GATA-3 (6, 7) and by ectopic expression of GATA-3, which induces Th2 cytokine production in Th1 cells and STAT6-deficient T cells (5, 8).

Interfering with GATA-3 function provides an additional mechanism of regulating the Th2 phenotype without altering GATA-3 expression levels. We have previously demonstrated that PU.1 interacts directly with GATA-3, which prevents the binding of GATA-3 to DNA and therefore antagonizes its function (9). PU.1 is an ETS family transcription factor that regulates various genes, including cytokine receptors required for the development of lymphoid and myeloid lineages (10). In Th2 cells, PU.1 is expressed in subpopulations that express low levels of IL-4 and effectively regulates the heterogeneity of the populations of Th2 cells (9). Decreasing PU.1 expression using a short hairpin RNA (shRNA) increases the homogeneity of Th2 cytokine production (9).

Although the cytokine milieu is a decisive component for T cell fate following T cell activation, additional factors contribute to the strength of the TCR signal and the degree of Th polarization (11). Among the factors that control the TCR signaling threshold are transcription factors such as IkAROS (12), costimulatory molecules such as B7 family members and LFA-1 (13, 14), Lck activity (15), and microRNAs that target multiple phosphatases to modulate TCR signaling (16). The strength of T cell activation is also determined by the expression of TCR and the amount of engagement of the complex (14). The ability of specific factors to modulate the expression of TCR genes in this context has not been well studied.

In this study, we found that T cells lacking PU.1 expression from mice with a conditional Sfpi1 allele had increased surface TCR expression on CD4+ T cells, resulting in increased T cell activation. Because GATA-3 was originally identified as a regulator for TCR expression (17), we hypothesized that PU.1 modulates the function of GATA-3, which regulates the levels of TCR expression. This regulation may play a critical role in setting the T cell activation threshold in a heterogeneous population of T cells.

Materials and Methods

Mice

Wild-type C57BL/6 female mice were purchased from Harlan Bioscience. Mice with conditional deletion of the PU.1 gene (Sfpi1lck+/−) on the C57BL/6 background were previously described (18) and mated to mice

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Received for publication February 3, 2009. Accepted for publication August 10, 2009.

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†This work was supported by U.S. Public Health Service Award AI057459 (to M.H.K.) from the National Institutes of Health.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900363

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carrying a Cre transgene under control of an Lck promoter (B6.CBA-Tg(Lck-cre)Fl540Xtm1J). Examination of allele deletion was performed as previously described (18). Mice were maintained in pathogen-free conditions and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

**CD4\(^+\) T activation**

CD4\(^+\) T cells were purified from spleens and lymph nodes as described above. CD4\(^+\) T cells (1 \times 10\(^{6}\) cells/ml complete RPMI 1640 medium) were cultured in a 6-well plate with coated anti-CD3 (clone 15S-2C11) ranging from 4 to 0.06 \(\mu\)g/ml and with soluble anti-CD28 (clone 37.51) at 0.5 \(\mu\)g/ml for 2 days. The supernatant was collected for determining the levels of IL-2 production, and the cells were analyzed for expression of activation markers using flow cytometry.

**Th cell differentiation**

CD4\(^+\) T cells were purified from spleens and lymph nodes as described above. CD4\(^+\) T cells (1 \times 10\(^{6}\) cells/ml complete RPMI 1640 medium) were cultured in a 6-well plate with coated anti-CD3 (2 \(\mu\)g/ml) and soluble anti-CD28 (0.5 \(\mu\)g/ml) mAbs against Th1 (IL-12 at 5 \(\mu\)g/ml Peprotech) and anti-IL-4 (11B11) mAb at 10 \(\mu\)g/ml or Th2 (IL-4 at 10 \(\mu\)g/ml Peprotech) and anti-IFN-\(\gamma\) (XMG) mAb at 10 \(\mu\)g/ml differentiation conditions. After 3 days of culture, 8 ml complete RPMI 1640 medium was added to each well. After 5–6 days of culture, cell counts were determined using a hemacytometer. Differentiated cells were restimulated with plate-bound anti-CD3 (1 \(\mu\)g/ml) and the cell-free supernatant was collected after centrifugation and stored at \(-20^\circ\)C until use. The levels of IL-2, IL-4, IL-5, IL-10, IL-13, and IFN-\(\gamma\) produced were determined using ELISA analysis (9).

Total protein extracts (100 \(\mu\)g/ml) of Th2 cells differentiated from both C57BL/6 and Sfpi1\(^{-/-}\) mice were used for DNA affinity precipitation assay (DAPA) as described previously (9). Cells were lysed with cell lysis buffer (19) and total protein extracts were separated on SDS-PAGE gel followed by immunoblot analysis with Abs against GATA-3 (R&D Systems) and GAPDH (Cell Signaling).

**Flow cytometry and cell sorting**

Cells from spleen and thymus of both C57BL/6 and Sfpi1\(^{-/-}\) mice were stained with anti-CD4 FITC and anti-CD8 PE Abs (BD Pharmingen). Double-negative (DN) thymocytes were sorted as CD4\(^+\) CD8\(^-\) and divided into populations based on CD25 and CD44 expression. The expression of activation markers were analyzed by staining the cells with anti-CD25 FITC and anti-CD69 PerCP Abs (BD Pharmingen). TCR expression was measured by staining the cells with anti-TCR\(\beta\) FITC (H57–597, BD Biosciences) and anti-CD3e PE (BD Biosciences). Cytokine intracellular staining was performed in differentiated Th2 cells that had been incubated in monensin for the last 2 hours of a 5-hour anti-CD3 stimulation (4 ng/ml). Stained cells were analyzed with the BD FACScalibur flow cytometer instrument. Total CD4\(^+\) T cells, expressing high (top 15\%) or low (bottom 15\%) TCR\(\beta\) levels, were purified by flow sorting using the BD FACSVantage SE.

**Retroviral transduction**

A bicistronic retroviral vector encoding mouse GATA-3 and human CD4 (hCD4) or hCD4 alone was described previously (9). Production of the retroviral supernatant followed by transduction into one-week-old differentiated Th1 cells was described previously (20). In transduced cells, expression of TCR\(\beta\) or CD3e was evaluated by flow cytometry on 5000 hCD4 positive cells. Transduction of differentiating Th2 cells with PU.1-expressing retrovirus was performed as previously described (9).

**Chromatin immunoprecipitation assay (ChIP)**

The ChIP experiment was performed using total CD4\(^+\) T cells or differentiated Th2 cells from wild-type and Sfpi1\(^{-/-}\) mice following the protocol as previously described (21). The resulting chromatin (2 \times 10\(^{6}\) cells per immunoprecipitation reaction) was immunoprecipitated using anti-GATA-3 (B6.H13-31) AC agarose (Santa Cruz Biotechnology) or normal mouse IgG-AC agarose, followed by washing, de-cross-linking, and DNA purification. Purified DNA was resuspended in double-distilled H\(_2\)O. The enriched DNA was analyzed for TCR enhancer (E) \(\beta\) (forward primer: 5'-TGT AGG ACC TGG TAA ATG TCA ACA A-3' and reverse primer: 5'-GGA AGG GGT GGA AGC ATC TC-3') (22), TCR Eα (forward primer: 5'-GCC AGA AAG TGG GAA GGA AAG TGA AO-3' and reverse primer: 5'-GGGAAGCTTGTGCGCATTGT-3') (23), and IL4 Va (forward primer: 5'-GAC TGA GAA CCC AAC AGA GAT GC-3' and reverse primer: 5'-GCC TGT GTT CCA TAT ATT CAT GAC T-3') (24) individually, using quantitative PCR in a SYBR Green Fast reaction (ABI 7500 Fast). The amount of PCR product was determined as percent of input relative to a standard curve of input chromatin. The ChIP results were calculated by subtracting the amount of DNA in the normal mouse IgG control from that in the anti-GATA-3 sample.

**Gata3 small interfering RNA (siRNA)**

Total CD4\(^+\) T cells (5 \times 10\(^{6}\) from wild-type and Sfpi1\(^{-/-}\) mice were transiently transfected with predesigned Gata3 siRNA (0.6 \(\mu\)M; Santa Cruz Biotechnology) and scrambled control (0.3 \(\mu\)M; Dharmacon) using the Amaxa Nucleofector (Lonza). After nucleofection, cells were rested in 5 ml complete medium in 5% CO\(_2\) incubator for 4 h. Cells (1 \times 10\(^{6}\)/ml) were cultured with plate-bound anti-CD3 at 1 \(\mu\)g/ml and soluble anti-CD28 at 0.5 \(\mu\)g/ml for 2 days. IL-2 levels were evaluated from supernatants using ELISA. GATA-3 protein levels were determined using Western blot.

**Statistics**

Data are presented as mean ± SD. Statistical significance was evaluated with an independent Student’s t test using SPSS 16.0 program (SPSS), and \(p < 0.05\) was considered significant.

**Results**

**Normal development of CD4\(^+\) T lymphocytes from Sfpi1\(^{-/-}\) mice**

We have previously demonstrated the function of PU.1 in Th2 cells using shRNA in cultured cells (9). To further analyze the effects of PU.1 deficiency on T cell phenotypes, we used mice that carry conditional alleles of the Sfpi1 gene on the C57BL/6 background and crossed to lck-Cre transgenic mice (denoted as Sfpi1\(^{+/--}\)) (18). The lck-Cre transgene mediates deletion of the floxed Cre allele beginning in the DN3 stage of thymocyte development (Fig. 1A). In naive CD4 T cells isolated from spleen the nondeleted allele was not detected (Fig. 1A). Importantly, deletion of the Sfpi1 allele is not complete until after Sfpi1 expression is extinguished from developing thymocytes (Fig. 1B) as previously described (18, 25). Thus, as expected, the T cell populations in Sfpi1\(^{+/--}\) mice as analyzed by flow cytometry were indistinguishable from wild-type mice. Flow cytometry of thymocyte populations showed similar profiles (p > 0.05) of CD4\(^+\) and CD8\(^+\) T cells between wild-type and Sfpi1\(^{+/--}\) mice (Fig. 1). The proportions of mature CD4\(^+\) and CD8\(^+\) T cells in the spleens of the Sfpi1\(^{+/--}\) mice were comparable to spleens from wild-type mice (p > 0.05; Fig. 1C). In addition, we did not observe any significant difference in the percentage of other populations examined, including T cells (CD3\(^+\)), B cells (B220\(^+\)), NK and NKT cells (CD3\(^+\) NK1.1), naive and memory CD4\(^+\) T cells (CD4\(^+\) CD62L\(^-\) CD44\(^+\)), or natural T regulatory cells (CD4\(^+\) CD25\(^+\)) (data not shown). The total numbers of thymocytes (11.5 ± 4 \times 10^7 in Sfpi1\(^{+/--}\) mice vs 13.3 ± 6 \times 10^7 in wild-type mice, n = 3) and splenocytes (8 ± 6 \times 10^7 in Sfpi1\(^{+/--}\) mice vs 4 ± 2 \times 10^7 in wild-type mice, n = 4) in Sfpi1\(^{+/--}\) mice did not differ (p > 0.05) from those of wild-type controls.

**PU.1 regulates T cell signaling threshold and Th2 cytokine production**

We next wanted to examine the sensitivity of CD4\(^+\) T cells in response to different strengths of stimuli using increasing concentrations of plate-bound anti-CD3e with a constant concentration of soluble anti-CD28. CD4\(^+\) T cells from Sfpi1\(^{+/--}\) mice produced significantly higher levels of IL-2 (p < 0.05) compared with cells from wild-type control mice, even at the lowest dose of anti-CD3 at 0.06 \(\mu\)g/ml following 48 h of stimulation (Fig. 2A). Similar results are observed using conditional PU.1-deficient T cells from mice expressing Cre from a CD4 promoter (data not shown), suggesting that increased IL-2 production is not due to an early thymic defect or a specific effect of the lck-Cre transgene. Although the
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FIGURE 1. T cell development in the absence of PU.1. A, Thymocytes or splenocytes were sorted into the indicated populations and genomic DNA was analyzed for the presence of the wild-type (WT; +), floxed (fl), or deleted (Δ) allele. Results are representative of two experiments. B, Thymocytes from Sfpi1<sup>flk−/−</sup> and wild-type control mice were sorted into DN subpopulations and RNA was analyzed for expression of Sfpi1. Results are averages ± SD of two independent experiments. C, Flow cytometry analysis of cells in thymus and spleens from Sfpi1<sup>flk−/−</sup> and wild-type control mice. The CD4<sup>+</sup> or CD8<sup>+</sup> T cell percentage is presented as mean ± SD of six mice from splenocytes and three mice from thymus. CD4<sup>+</sup> T cell development was normal in Sfpi1<sup>flk−/−</sup> mice as shown by similar (p > 0.05) percentage of cells staining with anti-CD4 and/or anti-CD8. DP, Double positive.

Percentage of cells expressing CD25 was similar between PU.1-deficient and wild-type cells 48 h after anti-CD3 stimulation, the expression of CD25 presented as mean fluorescent intensity (MFI) was significantly (p < 0.05) higher in CD4<sup>+</sup> T cells lacking PU.1 compared with those from wild-type cells (Fig. 2B and data not shown).

Because PU.1 appeared to modulate the activation threshold of T cells and because we have previously shown PU.1 to regulate Th2 cytokine production, we next evaluated the phenotype of Th2 populations in the absence of PU.1. Given the ability of PU.1 deficiency to increase the sensitivity of T activation, we compared the differentiation of wild-type and Sfpi1<sup>flk−/−</sup> CD4<sup>+</sup> T cells under optimal (2 μg/ml) or suboptimal (0.125 μg/ml) concentrations of anti-CD3 in combination with constant doses of anti-CD28, IL-4, and anti-IFN-γ for Th2 differentiation. After 5–6 days of culture, the numbers of Th2 cells from Sfpi1<sup>flk−/−</sup> mice were almost 2-fold higher than those from wild-type mice following suboptimal stimulation (Fig. 3A). However, the cell numbers did not differ significantly (p > 0.05) under optimal concentrations of anti-CD3 (Fig. 3A). The requirement for lower CD3 stimulation to observe significant differences between wild-type and Sfpi1<sup>flk−/−</sup> T cells may be due to higher expression of Sfpi1 in cells cultured with lower doses of anti-CD3 (Fig. 3B). T2 cells (1 × 10<sup>6</sup> cells/ml) differentiated under either condition were restimulated with anti-CD3 (2 μg/ml) or suboptimal (0.125 μg/ml) for 24 h. The levels of secreted cytokines in the supernatant were significantly (p < 0.05) higher in Th2 cultures from Sfpi1<sup>flk−/−</sup> mice compared with the wild-type control under the suboptimal concentrations of anti-CD3 (Fig. 3C). Although the levels of Th2 cytokine production under optimal anti-CD3 stimulation showed a similar trend of Th2 cytokines production, only IL-5 production was significantly different from wild-type cultures. The levels of IFN-γ in Th2 cultures were similarly (p > 0.05) low between Sfpi1<sup>flk−/−</sup> and wild-type control cultures stimulated with either optimal or suboptimal anti-CD3 (Fig. 3C). As stated above, greater differences between wild-type and Sfpi1<sup>flk−/−</sup> Th2 cells when cultured under the suboptimal conditions may be due to increased expression of PU.1 under these conditions (Fig. 3C).

We have previously shown that reduction of PU.1 expression by shRNA increases the homogeneity of Th2 cytokine production, as evidenced by more cells simultaneously producing two Th2 cytokines (9). We predicted that Th2 cells differentiated from Sfpi1<sup>flk−/−</sup> mice would be more homogenous in their cytokine production than wild-type cells. Indeed, intracellular cytokine staining revealed significantly (p < 0.05) more IL-4/IL-5 and IL-5/IL-10 double-positive-producing Th2 cells in the absence of PU.1 than those cells from wild-type control mice under the suboptimal anti-CD3 (0.125 μg/ml) stimulation (Fig. 4A). There were

FIGURE 2. CD4<sup>+</sup> T cells from Sfpi1<sup>flk−/−</sup> mice are more sensitive in response to stimuli. CD4<sup>+</sup> T cells isolated from Sfpi1<sup>flk−/−</sup> and wild-type (WT) control mice were stimulated with the indicated concentrations of plate-bound anti-CD3e and a constant concentration of soluble anti-CD28 (0.5 μg/ml) for 2 days. A, Supernatants were collected for determining the levels of IL-2 production using ELISA. B, The expression levels (MFI) of CD25 were evaluated using flow cytometry. Values presented are mean ± SD of three mice from each group. * Significant difference between wild-type control and Sfpi1<sup>flk−/−</sup> samples (p < 0.05). Results shown are representative of two independent experiments.
also significant increases ($p < 0.05$) in IL-5-single positive populations (Fig. 4A). However, there were similar ($p > 0.05$) frequencies of double-positive cytokine-producing cells when cultured under optimal anti-CD3 (2 μg/ml) stimulation conditions (Fig. 4B). In agreement with the ELISA results (Fig. 3B), IL-5 single-positive populations were also significantly higher under optimal conditions (Fig. 4B).

**PU.1 interferes with GATA-3-dependent TCR expression**

Because CD4$^+$ T cells are more sensitive to stimuli in the absence of PU.1 expression, we hypothesized that CD4$^+$ T cells

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**FIGURE 3.** Cytokine production from wild-type (WT) and PU.1-deficient Th2 cultures. Total CD4$^+$ T cells isolated from both Sfpi1$^{+/-}$ and wild-type control mice were differentiated into Th2 cells in the presence of either 0.125 or 2 μg/ml anti-CD3e. A, The numbers of differentiated cells were determined by hemocytometer with trypan blue staining. B, Sfpi1 mRNA was determined in wild-type Th2 cells differentiated with the anti-CD3 dose indicated using quantitative PCR. C, Differentiated Th2 cells were restimulated with plate-bound anti-CD3ε (2 μg/ml) for 24 h. The supernatant was collected for cytokine ELISA. Data presented are mean ± SD of three mice from each group. *, Significant difference between wild-type control and Sfpi1$^{+/-}$ samples ($p < 0.05$). Results shown are representative from five independent experiments. NS, Not significant.

**FIGURE 4.** Intracellular staining of cytokine production in wild-type (WT) and PU.1-deficient Th2 cells. Th2 cells were differentiated with anti-CD3 at 0.125 μg/ml (A) or 2 μg/ml (B), restimulated with plate-bound anti-CD3ε (4 μg/ml), and treated with monensin for the last 2 h of a 5-h incubation. Intracellular cytokine production was determined by staining cells with fluorochromes-conjugated Abs including IL-4, IL-5, and IL-10. Results are presented as mean ± SD of three mice from each group. *, Significant difference between wild-type control and Sfpi1$^{+/-}$ samples ($p < 0.05$). Results shown are a representative from five independent experiments.
from *Sfpi*<sup>lck<sup>−/−</sup> mice had higher levels of TCR expression, which contributed to increased sensitivity to anti-CD3 stimulation. We examined the levels of TCR expression from freshly isolated splenocytes. Flow cytometry revealed that both CD3e and TCRβ (clone H57–597) expression were significantly (*p* < 0.05) higher in T cells isolated from *Sfpi*<sup>lck<sup>−/−</sup> mice than those from wild-type mice (Fig. 5A). We also observed that individual TCR Vβ regions (mouse Vβ TCR screening panel; BD Biosciences) on T cells from *Sfpi*<sup>lck<sup>−/−</sup> mice had higher expression levels than wild-type cells (data not shown). CD3 and TCRβ expression were also higher on CD4 single-positive thymocytes from *Sfpi*<sup>lck<sup>−/−</sup> mice than wild-type cells and, in spleen, CD3 and TCRβ expression were higher on both naive and memory phenotype CD4 T cells (Fig. 5, B and C, and data not shown). As increased TCR expression might explain the increased sensitivity to Ag receptor stimulation (Fig. 2), we next tested whether IL-2 production is different when bypassing the TCR complex by stimulating wild-type and *Sfpi*<sup>lck<sup>−/−</sup> T cells with PMA and ionomycin. IL-2 production was similar between wild type and *Sfpi*<sup>lck<sup>−/−</sup> cells with PMA and ionomycin, suggesting that the increased sensitivity of *Sfpi*<sup>lck<sup>−/−</sup> T cells to anti-CD3 stimulation is due to altered Ag receptor expression and not other alterations in downstream signaling factors (Fig. 5D).

**FIGURE 5.** TCR expression on wild-type (WT) and *PU.1*<sup>−/−</sup> deficient CD4<sup>+</sup> T cells. A–C, CD4<sup>+</sup> splenocytes (A), CD4 single-positive thymocytes (B), or CD4<sup>+</sup> splenocytes gated on naive (CD62L<sup>high</sup> CD44<sup>low</sup>) or memory (CD62L<sup>low</sup> CD44<sup>high</sup>) populations (C) isolated from *Sfpi*<sup>lck<sup>−/−</sup> and wild-type mice were evaluated for the surface expression of CD3e and TCRβ as indicated. The expression levels are presented as mean MFI ± SD of three mice from each group. D, CD4 T cells from isolated from *Sfpi*<sup>lck<sup>−/−</sup> and wild-type splenocytes were stimulated with PMA (10 or 50 ng/ml as indicated) and ionomycin (500 ng/ml) for 48 h before IL-2 concentration in supernatants was determined using ELISA. E, CD4<sup>+</sup>CD62L<sup>−</sup> (naive) T cells were isolated using magnetic selection. Total protein extracts were subjected to Western blotting analysis for GATA-3 with GAPDH as a loading control. The Western blot band intensity was analyzed by densitometry and normalized to GAPDH as the ratio of GATA-3 and GAPDH. Data are presented as the mean ratio ± SD of two duplicates. F, Isolated total CD4<sup>+</sup> T cells were pooled from three wild-type and three *Sfpi*<sup>lck<sup>−/−</sup> mice for ChIP assay to evaluate the binding of GATA-3 to the TCR E<sub>β</sub> region. The DNA amplified by each primer set using real-time PCR is presented as the mean percentage of input ± SD of two duplicates. G, CD4 T cells isolated from *Sfpi*<sup>lck<sup>−/−</sup> and wild-type splenocytes were transfected with Gata3-specific siRNA and stimulated with anti-CD3 (1 μg/ml) for 48 h before determination of IL-2 concentrations in supernatants using ELISA. Inset, Immunoblot of GATA-3 and GAPDH in control (Ctrl) and Gata3 siRNA transfected cells. Numbers indicate the relative level of GATA-3 protein expression were also higher on CD4 single-positive thymocytes from *Sfpi*<sup>lck<sup>−/−</sup> mice (data not shown). As increased TCR expression might explain the increased sensitivity to Ag receptor stimulation (Fig. 2), we next tested whether IL-2 production is different when bypassing the TCR complex by stimulating wild-type and *Sfpi*<sup>lck<sup>−/−</sup> T cells with PMA and ionomycin. IL-2 production was similar between wild type and *Sfpi*<sup>lck<sup>−/−</sup> T cells stimulated with PMA and ionomycin, suggesting that the increased sensitivity of *Sfpi*<sup>lck<sup>−/−</sup> T cells to anti-CD3 stimulation is due to altered Ag receptor expression and not other alterations in downstream signaling factors (Fig. 5D).
GATA-3 was originally identified as an important transcription factor for regulation of TCRα gene expression (17). In addition, PU.1 antagonizes GATA-3 function by direct interaction and prevents the binding of GATA-3 to target genes (9). The average level of GATA-3 protein expression in naive CD4 T cells from Sfpi1+/− mice was not significantly (p > 0.05) different from wild-type controls (Fig. 5E). However, the binding of GATA-3 to the TCR Eβ region in total CD4+ T cells, as evaluated by ChIP assay, was increased 95% in T cells from Sfpi1+/− mice compared with wild-type cells (Fig. 5F).

To determine whether GATA-3 was required for increased IL-2 production from Sfpi1+/− T cells, activated wild-type and Sfpi1+/− T cells were transfected with control or Gata3-specific siRNA. Diminishing GATA-3 expression decreased IL-2 production from wild-type cells and also reduced IL-2 production from Sfpi1+/− T cells to amounts near control wild-type T cells (Fig. 5G). Thus, GATA-3 is required for the increased sensitivity of Sfpi1+/− T cells to Ag receptor stimulation.

Because we observed higher Th2 cytokine production in cultures differentiated from Sfpi1+/− mice (Figs. 3 and 4), whereas the levels of GATA-3 protein were similarly expressed in differentiated wild-type and PU.1-deficient Th2 cultures (Fig. 5H), we hypothesized that the DNA binding activity of GATA-3 was enhanced in Th2 cells lacking PU.1. To determine whether there was an increase in total GATA-3 DNA binding activity, we tested GATA-3 DNA binding activity using DAPA. DAPA experiments revealed that 60% more GATA-3 was capable of binding DNA from PU.1-deficient extracts than from wild-type extracts (Fig. 5H). Similarly, increased binding activity of GATA-3 to TCR Εα and Εβ regions was observed using ChIP assay with Th2 cells differentiated from Sfpi1+/− mice compared with wild-type controls (Fig. 5F). As we previously observed in IL-4′ cells (9), PU.1 expression limited GATA-3 binding to the Il4 Va enhancer (Fig. 5I). Thus, PU.1 limits GATA-3 DNA binding to Tcr and Il4 loci.

Our results suggest that the increased DNA binding activity of GATA-3 in Sfpi1+/− mice contributes to higher levels of TCR expression. This further suggests that Th2 cells, which have higher expression of GATA-3 than Th1 cells, might also express higher TCR levels. Following differentiation, Th2 cells express almost 20-fold higher levels of GATA-3 compared with Th1 cells, with a reciprocal pattern of T-bet expression (Fig. 6A). Flow cytometry analysis revealed that TCR expression was higher in differentiated Th2 cultures than in Th1 cells (Fig. 6B). In addition, levels of Tcra mRNA were higher in Th2 cells than Th1 cells (Fig. 6C).

We hypothesized that altering the levels of GATA-3 activity would affect TCR expression. We first tested whether ectopic expression of GATA-3 in Th1 cells would increase TCR expression. After 6 days of differentiation, Th1 cells from wild-type mice were transduced with retroviruses encoding GATA-3 and human CD4 (hCD4) or hCD4 alone. We have previously shown that GATA-3 transduction into differentiated Th1 does not eliminate the Th1 phenotype (20). Three days after transduction, expression of TCRβ and CD3ε were evaluated on hCD4-positive cells. Flow cytometry showed that ectopic expression of GATA-3 in differentiated Th1 cells increased TCR expression (Fig. 6D). We then tested the converse; whether increased PU.1 expression would decrease TCR expression. Differentiating Th2 cells were transduced with control or PU.1-expressing retroviruses and sorted for enhanced GFP expression (9). Ectopic PU.1 expression decreased Tcra mRNA levels compared with control transduced cells (Fig. 6E). These results further supported that higher levels of GATA-3 activity contributed to increased TCR expression.

T cells exhibit heterogeneous TCR expression as indicated by the distribution of TCR staining in flow cytometry (Fig. 7A). We purified the cell populations expressing high (MFI = 100) and low (MFI = 25) levels of TCRβ for further analysis. After flow sorting from wild-type control mice, TCR-low and TCR-high populations were stimulated with increasing doses of anti-CD3. Both TCR-low and TCR-high cells were activated upon stimulation because CD69 was expressed on both populations with a slightly higher intensity in TCR-high populations (Fig. 7B). However, TCR-high populations were more sensitive to increasing concentrations of anti-CD3 stimulation, producing much higher levels of IL-2 than similarly stimulated TCR-low cells (Fig. 7C). Although TCR-high and TCR-low populations expressed similar levels of GATA-3 (Fig. 7D), TCR-low CD4 T cell populations had almost 100-fold higher expression of TCRα compared with Th1 cells (Fig. 7E). Similarly, in Th2 cells, TCR-low cells had higher expression of Sfpi1 and lower expression of Il4- (Fig. 7, E and F), consistent with our previous observations (9). Together, these data indicate that PU.1 interferes with GATA-3-dependent regulation of TCR expression.

**Discussion**

We have previously demonstrated that PU.1 expression affects the heterogeneity of Th2 phenotypes by antagonizing GATA-3-DNA binding activity and therefore regulates the levels of Th2 cytokine expression (9). In this study, we further evaluate the function of
PU.1 in Th2 cells using mice with a conditional deletion of the PU.1 gene. Results indicate that PU.1 modulates the levels of TCR expression in CD4+ T cells by regulating the DNA-binding activity of GATA-3. In the absence of PU.1 in Sfpi1−/− cells, increased GATA-3 function resulted in increased TCR expression, lowering the activation threshold and making CD4+ T cells more sensitive to anti-CD3 engagement and activation. The increased activation threshold is dependent upon TCR ligation because IL-2 production was similar between wild-type and Sfpi1−/− T cells stimulated with PMA/ionomycin. GATA-3 is required for this effect and Gata3-specific siRNA reduced IL-2 production from PU.1-deficient T cells.

The development of hematopoietic cells is directed by the activity of lineage-specific transcription factors that are expressed at different concentrations during a particular window of time. During T cell development, the level of PU.1 expression is greatly reduced from the DN2 to the DN3 stages of pro-T development (Fig. 1B), which is required for T lineage commitment in the pre-T stage. Enforced expression of PU.1 in fetal hematopoietic progenitors arrests T cells at the pro-T stage (25). In Sfpi1−/− mice, ablation of PU.1 expression begins at the DN3 stage (Refs. 26 and 27, and Fig. 1A), making deletion unlikely to interfere with normal T cell development. Deletion of the allele was highly efficient because we did not detect the nondeleted allele in naive CD4 T cells (Fig. 1A). As such, we did not observe any defects in T cell development in Sfpi1−/− mice (Fig. 1). In addition, GATA-3 expression is similar in naive CD4+ T cells isolated from wild-type and Sfpi1−/− mice (Fig. 5E), suggesting that lacking PU.1 expression from DN3 on does not perturb the balance of the transcriptional environment during the T cell development.

Regulation of TCR expression by GATA-3 has been previously described. GATA-3 is required at multiple stages of T cell development, including the early DN stages, β-selection between DN3 and DN4, and positive selection of CD4 single-positive thymocytes (28). Enforced GATA-3 expression in CD2-GATA-3 transgenic mice resulted in enhanced TCR up-regulation at the double-positive and CD4 single-positive thymocytes (29, 30). In addition, GATA-3 deficiency in CD4-Cre Gata3fl/fl mice is associated with diminished expression of surface TCR in single-positive CD4 and CD8 thymocytes (6, 29). In this study, we identified PU.1 as a regulator GATA-3 function. PU.1 deficiency results in increased TCR expression and reduced activation thresholds in mature CD4+ T cells (Figs. 5 and 7). Moreover, the heterogeneity of TCR expression correlates with PU.1, in that PU.1 expression is higher in TCR-low T cells (Fig. 7). It is possible that PU.1 could also have direct effects on TCR expression. Other ETS family members can activate TCR gene expression when they bind directly to regulatory elements, though it is not clear whether these factors are required for TCR gene expression (31–33). Our data suggests that regulating GATA-3 activity may be an additional mechanism of controlling T cell activation.

Transgenic models have demonstrated that Ag dose contributes to the polarization of T cells (11). Similarly, the regulation of TCR expression and activation thresholds by PU.1 contributes to the development of Th2 cells. In Th2 polarizing conditions where TCR engagement was limiting, the numbers of Th2 cells and the cytokine production from Th2 cells were increased in Sfpi1−/− cultures compared with wild-type cultures under the same conditions. However, when TCR engagement was not limiting, the differences in Th2 cytokine production between wild-type and Sfpi1−/− cultures were minimized. This is also reflected in Th2 homogeneity, defined as cells producing two or more cytokines simultaneously (9). In conditions of limiting TCR engagement, more Th2 cells are double-cytokine producers from Sfpi1−/− cultures than from wild-type cultures (Fig. 4A). This suggests that lowering the activation threshold by deleting PU.1 expression increased Th2 cell homogeneity. The differences between wild-type and Sfpi1−/− cells may be more dramatic in low-dose anti-CD3...
cultures because Sfpi1 expression is increased under these conditions, making the effects of deficiency more apparent. Thus, PU.1 may be regulating Th2 heterogeneity through several mechanisms. First, it regulates GATA-3 activity in naive CD4 T cells, setting a threshold of activation that ultimately impacts the differentiation phenotype. The proportion of naive CD4 T cells expressing PU.1 is still unclear. Our previous report demonstrated that PU.1 is expressed in naive cells, and expression is further induced by activation (9). Our data suggests that only a subpopulation of T cells expresses PU.1 and in those cells TCR expression is the lowest (Fig. 7). It is also possible that a gradient of PU.1 expression exists in all naive T cells and that in PU.1-deficient cells, TCR expression of the entire population shifts up, rather than changes of expression in only a subpopulation of cells. At the moment it is hard to distinguish between these possibilities. In the subpopulation of Th2 cells that express PU.1, GATA-3 DNA binding is limited and the activation of Th2 cytokine loci is decreased. Both of these mechanisms may be important in determining the potency of the developing Th2 response and the function of the Th2 cells that develop.

T cells are activated upon engagement of the TCR-CD3 complex, which triggers the signaling events of several ITAM motifs on CD3 subunits (34). The number of expressed TCR on the surface, which triggers the signaling events of several ITAM motifs, may be important in determining the potency of the developmental pathways. The proportion of naive CD4 T cells expressing PU.1, GATA-3 DNA binding is limited and the activation of Th2 cytokine loci is decreased. Both of these mechanisms may be important in determining the potency of the developing Th2 response and the function of the Th2 cells that develop.

Acknowledgments

We thank Dr. A. Dent for critical review of this manuscript and the staff of the Indiana University Simon Cancer Center Flow Cytometry Facility.

Disclosures

The authors have no financial conflict of interest.

References

1. Kaplan, M. H., U. Schindler, S. T. Smiley, and M. J. Grusby. 1996. Stat6 is required for signaling events of several ITAM motifs on CD3 subunits (34). The number of expressed TCR on the surface determines the magnitude of T cell activation (14). The higher the TCR expression on the surface of T cells, the lower activation threshold they require for activation, and thus are more sensitive to stimulation. In this report, we have identified PU.1 as a regulator of the entire population shifts up, rather than changes of expression in only a subpopulation of cells. At the moment it is hard to distinguish between these possibilities. In the subpopulation of Th2 cells that express PU.1, GATA-3 DNA binding is limited and the activation of Th2 cytokine loci is decreased. Both of these mechanisms may be important in determining the potency of the developing Th2 response and the function of the Th2 cells that develop.

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