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IFN Regulatory Factor 4 Regulates the Expression of a Subset of Th2 Cytokines

Ayele-Nati N. Ahyi, Hua-Chen Chang, Alexander L. Dent, Stephen L. Nutt, and Mark H. Kaplan

Th2 cells can be subdivided into subpopulations depending on the level of a cytokine and the subsets of cytokines they produce. We have recently identified the ETS family transcription factor PU.1 as regulating heterogeneity in Th2 populations. To define additional factors that might contribute to Th2 heterogeneity, we examined the PU.1 interacting protein IFN-regulatory factor (IRF)4. When Th2 cells are separated based on levels of IL-10 secretion, IRF4 expression segregates into the subset of Th2 cells expressing high levels of IL-10. Infection of total Th2 cells, and IL-10 nonsecreting cells, with retrovirus-expressing IRF4, resulted in increased IL-4 and IL-10 expression, no change in IL-5 or IL-13 production and decreased IL9 transcription. Transfection of an IRF4-specific small interfering RNA into Th2 cells decreases IL-10 production. IRF4 directly binds the IL10 gene as evidenced by chromatin immunoprecipitation assay, and regulates IL10 control elements in a reporter assay. IRF4 interacts with PU.1, and in PU.1-deficient T cells there was an increase in IRF4 binding to the IL10 gene, and in the ability of IRF4 to induce IL-10 production compared with wild-type cells and IL10 promoter activity in a reporter assay. Further heterogeneity of IRF4 expression was observed in Th2 cells analyzed for expression of multiple Th2 cytokines. Thus, IRF4 promotes the expression of a subset of Th2 cytokines and contributes to Th2 heterogeneity. The Journal of Immunology, 2009, 183: 1598–1606.
μg/ml anti-IFN-γ (R6/62A or XMG) for Th2 differentiation, or 10 ng/ml IL-12 plus 10 μg/ml anti-IL-4 (11B11) for Th1 differentiation. After 3 days of incubation, cells were expanded for a total of 5 or 6 days. Differentiated cells were restimulated with 2 μg/ml anti-CD3 at a concentration of 10^6 cells/ml for real-time PCR and ELISA as previously described (8, 22). Statistics were performed using a t test with SPSS software (v. 16.0; SPSS).

**Immunoprecipitation and Western blot analysis**

Total cell extracts were prepared by lysing Th2 cells with lysis buffer (10% glycerol, 1% Igepal, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8)) for 15 min on ice before centrifugation at 14,000 rpm for 15 min at 4°C. Nuclear and cytoplasmic proteins were prepared from differentiated Th2 cells using Nuclear and Cytoplasmic Extraction Reagents from Pierce Biotechnology. Protein extracts (25 μg) from naïve T, Th1, Th2, and Pho- niex cells were separated on 4–12% SDS-PAGE and transferred onto Ny- tron membranes (Schleicher and Schuell Bioscience). Immunoprecipitation was performed as previously described (8). Nuclear cell lysates (1 mg Th2 extracts) were incubated with the nuclear protein for 15 min at room temperature before the addition of the biotinylated oligonucleotide was incubated with the nuclear protein for 15 min at room temperature before the addition of the biotinylated IL-10 promoter oligonucleotide. The IRF4 consensus binding site was deleted in the IRF4 mut- tant competitor oligonucleotide and the sequence is TGAGGTCTAT- CAGCCC TCTCGGG and the reverse complement. The sequence for the GATA-3 conjugated with protein G beads (Santa Cruz Biotechnology) overnight at 4°C before precipitation with protein G beads. Immunocomplexes were separated by SDS-PAGE and immunoblots were probed with the precipitating Abs.

**DNA affinity precipitation assay**

Th2 nuclear lysate (250 μg) was incubated with double stranded bionytin- alized oligonucleotides as described (23). The sequences for B-I promoter oligonucleotide are biotin-TGAGGTCTGAAAGAATCACGCCCCTCG GG and the reverse complement. For the competition assay, the competitor oligonucleotide was incubated with the nuclear protein for 15 min at room temperature before the addition of the biotinylated IL-10 promoter oligonucleotide. The IRF4 consensus binding site was deleted in the IRF4 mut- tant competitor oligonucleotide and the sequence is TGAGGTCTAT- CAGCCC TCTCGGG and the reverse complement. The sequence for the GATA-3 conjugated with protein G beads (Santa Cruz Biotechnology) was previously de- scribed (8, 24). Protein-DNA complexes were separated by SDS-PAGE and immunoblots were probed with anti-IRF4.

**Retroviral vectors and transduction**

The retroviral vector MIEG-hCD4 was previously described (8). The coding region for human IRF4 cDNA was amplified by PCR and cloned into MIEG-hCD4. The Phoenix GP packaging cells line was transiently trans- fected with 15 μg of plasmid DNA. Transduced Th2 cells were lysed with reporter lysis buffer (Promega) and the luciferase and β-galactosidase activity of the sample. The luciferase activity was normalized to the β-galactosidase activity. The NFAT reporter is a trimer of CAGCCC TCTCGGG and the reverse complement. The sequence for the IRF-4 competitor and GATA-3 oligonucleotide was previously de- scribed and characterized in culture, we cultured CD11c^+ cells as described above, the mRNA is 9-fold

**Flow cytometry**

Cells were restimulated with 4 μg/ml α-CD3 for 3 h before 3-h treatment with 3 μM monensin. Intracellular cytokine staining was performed using fluorochrome-conjugated Abs (BD Pharmingen) and α-IL-4 PECy7 (eBioscience). IRF4 staining was performed using α-IRF4 (Santa Cruz Biotechnol- ogy) and the secondary donkey anti-goat Ab (Jackson ImmunoResearch Laboratories) conjugated with Cy5 (Cyanine 5). Stained cells were ana- lyzed with an LSRII instrument.

**Isolation of IL-10 high and low populations**

CD4^+ T cells were differentiated into Th2 cells for 7 to 10 days as described above. The day before sorting, the differentiated cells were harvested counter and plated at 10^6 cells/ml in RPMI 1640 for 6 h. After this resting period, the cells were restimulated for 6 h with 10 ng/ml PMA and 1 μg/ml ionomycin. Cells were harvested and washed with MACs buffer. The IL-10 secreting and nonsecreting populations were labeled using the mouse IL-10 Secretion Assay (Miltenyi Biotec) before sorting with FACSVa aSive VE or FACSaria from Becton Dickin- son. The cells were rested for 1 or 2 days before restimulation, anal- ysis, or transduction.

**IL-10 high and low Th2 cocultures**

IL-10 high and low Th2 (1 × 10^6) cells were sorted as described above and cocultured with either 0.5 × 10^6 splenic CD11c^+ cells purified by positive selection using magnetic beads (Miltenyi Biotech) or 1 × 10^6 total spleno- cytes from BALB/c mice. Cells supplemented with RPMI 1640 medium were activated with 5 μg/ml anti-CD3 and 4 μg/ml LPS. dendritic cell coculture was harvested after 3 days for IFN-γ and IL-6 ELISA and total splenocyte coculture was harvested after 5 days for IgG1 ELISA.

**Chromatin immunoprecipitation assay (ChIP)**

CD4^+ T cells cultured under Th2 conditions for 5 days were fixed with formaldehyde to cross-link protein-DNA complexes and 5 × 10^6 cells were used per ChIP reaction. The ChIP assay was performed essentially as described (25) except that cells were resuspended in nuclear lysis buffer (50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 1% SDS, and protease inhibitor) at 4°C for 15 min before lysis on ice with lysis buffer (0.25 M LiCl, 1% Igepal, 1% sodium deoxycholate, 1 mM EDTA (pH 8.0), and 10 mM Tris (pH 8.0)), and two Tris-EDTA buffer washes (1 mM EDTA and 10 mM Tris (pH 8.0)). Real-time PCR was done with 2 μl (1.7 × 10^5 cells) of immunoprecipitated DNA from the same cells. Control IgG ChIP results are subtracted from specific Ab ChIP results and results are shown as the mean specific binding of replicates ± SD. Primers for Il10 and Il4 ChIP have been previously described (8, 26). Primers for the Il9 promoter were CAG TCT ACC AGC ATC TTC CAG TCT AGC and GTG GCC ACT GGG TAT CAG TTT GAT GTC.

**Reporter assay**

EL4 cells were cultured for 3 days before transfection of 10^6 cells by Amaza nucleofection or by electroporation with expression plasmid, re- porter vector containing the regulatory sequence and β-galactosidase in DMEM. Cells were immediately transfected to six-well plates. After 24 h, the cells were harvested, washed with PBS, and cultured in the presence or absence of 0.2 μg/ml ionomycin and 20 ng/ml PMA for 24 h. Harvested cells were lysed with reporter lysis buffer (Promega) and the luciferase activity was measured for each sample and divided by the total protein concentra- tion and the β-galactosidase activity of the sample. The Il4 and Il10 reporters were described previously (26). The NFAT reporter is a trimer of a distal Il2 promoter-binding site and was generously provided by Gerald Crabtree (Stanford University, Stanford, CA).

**Results**

**IRF4 expression defines IL-10 low and IL-10 high phenotype**

Having established a role for PU.1 in regulating Th2 heterogene- ity, we wanted to determine whether other factors required for Th2 differentiation also contribute to the expression of subsets of Th2 cytokines. We separated Th2 cultures into IL-4 high and low, and IL-10 high and low populations and examined the expression of Th2-associated transcription factors in each population. As previ- ously described, PU.1 segregated into the IL-4 low population (8). IL-10 high cells expressed 15-fold more Il10 mRNA and secreted 50-fold more IL-10 than IL-10 low cells (Fig. 1, A–C). Il4 expres- sion was 2-fold higher in IL-10-high than in IL-10-low cells. The IL-10-high and low phenotypes were stable as there was still a significant difference in IL-10 production from these cells after an additional week in culture (Fig. 1B). In contrast to the enrichment for IL-4 and IL-10 production, the level of Il9 mRNA is 9-fold higher in IL-10-low than in IL-10 high cells (Fig. 1D). To determine whether IL-10 high and low cells had different functional characteristics in culture, we cultured CD11c^+ splenocytes or total splenocytes in the absence or presence of either IL-10 high or low Th2 cells. We observed that IL-10 low cells stimulated increased IL-6 and IFN-γ production from LPS-stimulated dendritic cells, while IL-10-high cells has less of an effect on IL-6 production and
did not affect IFN-γ production (Fig. 1E). Conversely, IL-10-high cells, but not IL-10-low cells were able to induce class switching to IgG1 in LPS-stimulated splenocyte cultures (Fig. 1F). These data suggest that IL-10 high and low populations in Th2 cultures represent distinct states that have the potential for separate biological functions in vivo.

To determine whether there were transcription factors that were associated with differences in IL-10 expression, we screened IL-10 high and low cells for expression of a number of factors associated with regulating the Th2 phenotype, including Sfpi1, Maf, Bcl6, Zfp511, Zbtb32, Runx1, and Irf4. We observed that Irf4 expression was enriched 5-fold in the IL-10 high population (Fig. 1G). To confirm that IRF4 protein was also differentially expressed between IL-10 high and low cells, we performed intracellular staining for IL-10 and IRF4. There was an 8-fold difference in expression of IRF4 in cells gated for IL-10 high or IL-10 low expression
produced differentiating Th1 cell with control or IRF4-expressing retrovirus. To further demonstrate that IRF4 functioned in Th1 cells to increase IL-10 production, we transduced differentiating Th2 cells on the second day of a 5-day culture period. Cells sorted for hCD4 expression were restimulated with anti-CD3 and evaluated for cytokine production using ELISA. Ectopic expression of IRF4 in Th2 cells increased production of IL-10 and IL-4 by 8-fold and 4-fold, respectively, with no significant effect on IL-5 and IL-13 (Fig. 2B).

Because transduction of IRF4 increases specific cytokines in differentiating Th2 cells, we next tested whether it would alter the phenotype of IL-10 low cells isolated from differentiated Th2 populations. To assess the change in IL-10 low phenotype, we sorted IL-10-low cells from cells cultured under Th2 conditions for 10 days as described in Materials and Methods. IL-10 low cells were transduced with IRF4-expressing retrovirus and cultured for 2 days before selection and stimulation with anti-CD3 to assess the level of cytokine production. Transduction of IRF4 in IL-10 low cells enhanced the production of IL-10 and IL-4 by 6-fold and 4-fold, respectively, with no significant effect on IL-5 (Fig. 2C). These results demonstrate that IRF4 specifically increases IL-4 and IL-10 production from Th2 cells but does not induce other Th2 cytokines.

To further show that IRF4 is required for IL-10 expression in Th2 cultures, we differentiated Th2 cultures in vitro and transduced cells with IRF4-specific or scrambled siRNA. Because Irf4 is expressed at high levels in effector Th2 cells, the IRF4-specific siRNA resulted in only a partial reduction of Irf4 expression (Fig. 2D). However, there was a commensurate decrease in IL-10 production from cells transduced with IRF4-specific siRNA (Fig. 2D).

Th1 cells also secrete IL-10, though at levels much lower than those produced by Th2 cells and with fewer IL-10^+^ cells in the Th1 population than Th2 cultures, though most IL-10^+^ cells are also IFN-γ^+^ (Fig. 3A and data not shown) (27, 28). As IRF4 is also expressed in Th1 cells (Fig. 3B), we wanted to determine whether IRF4 expression also segregated with IL-10 expression in Th1 cultures. Intracellular staining for IL-10 and IRF4 in Th1 cells was similar to the pattern in Th2 cells where higher IRF4 was observed in IL-10 high cells (Fig. 3C). To further demonstrate that IRF4 functioned in Th1 cells to increase IL-10 production, we transduced differentiating Th1 cell with control or IRF4-expressing retrovirus. IL-10 transcription was then assessed using qPCR. IRF4 transduction increases production of specific Th2 cytokines. A, Schematic of control and bicistronic retroviral vectors containing cDNAs for human IRF4 (hIRF4) and human CD4 (hCD4, selectable marker). B, BALB/c CD4 T cells were transduced with control (MIEG) or IRF4 containing retroviruses on the second day of a 5-day culture under Th2 conditions. HCD4-positive cells were sorted and stimulated with anti-CD3. Supernatants were analyzed for concentration of the indicated cytokines using ELISA. C, CD4 T cells cultured under Th2 conditions for 7 days and sorted for low IL-10 secretion were transduced with control or IRF4-expressing retrovirus and cultured for an additional 3 days. HCD4-positive cells were then sorted and stimulated with anti-CD3. Supernatants were analyzed for concentration of the indicated cytokines using ELISA. D, CD4^+^ T cells cultured under Th2 condition for 5 days were transduced with scrambled siRNA or IRF4 siRNA. After 40 h, cells were either stimulated with anti-CD3 for 2 h and RNA was isolated for qPCR analysis of Irf4 mRNA or stimulated for 1 day and supernatants were tested for IL-10 concentration using ELISA.
IRF4 was previously shown to transactivate a reporter gene with the Il4 promoter (17), which we also observed in this study (Fig. 4D). To determine whether IRF4 could transactivate gene expression from the Il10 regulatory elements, we used a luciferase reporter containing either the Il10 promoter region or the Il10 CNS3 region, both of which contain IRF4 binding sites (Fig. 4A) (26). Upon transfection of EL4 cells with either of the Il10 reporters and IRF4-expressing or control pCEP4 vectors, the transactivation was measured by assessing luciferase activity. Cotransfection of IRF4 induced increased luciferase activity from promoter and CNS3 reporters (Fig. 4D). Stimulation of cells with PMA plus ionomycin increased basal reporter activity and IRF4 cotransfection was able to further increase reporter activity (Fig. 4D). As a negative control, IRF4 did not activate an NFAT reporter plasmid (Fig. 4D). Thus, IRF4 binds and directly transactivates Il10 regulatory elements.

Functional interactions of PU.1 and IRF4

PU.1 was demonstrated to interact with GATA binding protein-3 (GATA-3) using recombinant protein binding assays (29) and in Th2 cell coimmunoprecipitates (8). PU.1 is also known to interact with IRF4 in B cells (10, 12, 24, 30, 31). To investigate the interaction of PU.1 and IRF4 in Th2 cells, we used anti-PU.1, anti-IRF4, or anti-GATA-3. Immunoprecipitation with anti-GATA-3 confirmed the interaction between PU.1 and GATA-3, though little IRF4 was precipitated with this complex (Fig. 5A). Thus, although PU.1 interacts with IRF4 and GATA-3, these data suggest that PU.1-IRF4 and PU.1-GATA-3 are largely separate complexes, as little IRF4 was associated with GATA-3. Because IRF4 expression segregates in IL-10 high cells, these interactions would only be meaningful if PU.1 were also expressed in the same cells. To test this, we examined expression of Sfp1, encoding PU.1, in IL-10 high and low cells and observed that Sfp1 mRNA was enriched in the IL-10-high population in a pattern similar to Irf4 expression (Fig. 5B). Because IL-10 high cells are comprised of both IL-4 high and low cells, this does not contradict previous data showing a segregation of PU.1 expression in IL-4 high and low populations.

To determine whether the association of IRF4 and PU.1 had functional consequences, we used mice carrying a conditional allele of the Sfp1 gene, crossed to lck-Cre transgenic mice (denoted as Sfp1<sup>fl/fl</sup>-<sup>cre</sup>). We then compared the function of IRF4 in WT and Sfp1<sup>fl/fl</sup>-<sup>cre</sup> Th2 cells. ChIP demonstrated that IRF4 binding to the Il10 promoter is greater in Sfp1<sup>fl/fl</sup>-<sup>cre</sup> Th2 cells than in C57BL/6 Th2 cells (Fig. 5C). A similar trend of IRF4 binding was observed in Il4 promoter (data not shown). The level of Il4 DNase hypersensitivity site V<sub>x</sub> (32, 33) in the IRF4 precipitates was also greater in Sfp1<sup>fl/fl</sup>-<sup>cre</sup> Th2 cells than in WT Th2 cells though binding to Il10 CNS3 was only modestly affected by PU.1 deficiency. Th2 cultures from Sfp1<sup>fl/fl</sup>-<sup>cre</sup> mice produced slightly more IL-10 than WT cultures, supporting observations in our previous report (8). Concomitant with increased IRF4 binding to the Il10 locus, transduced IRF4 induced more IL-10 in Sfp1<sup>fl/fl</sup>-<sup>cre</sup> Th2 cells than in WT Th2 cells. We did observe that IRF4 transduction had a less robust increase in IL-10 production in C57BL/6 background cells, compared with BALB/c Th2 cells (Fig. 5D vs Fig. 2B) although IRF4 expression as assessed by flow cytometry was similar between BALB/c and C57BL/6 cells, and between C57BL/6 WT or Sfp1<sup>fl/fl</sup>-<sup>cre</sup> cells (data not shown). To further demonstrate the ability of PU.1 to interfere with gene activation by IRF4 we repeated the reporter assay in Fig. 4D with the

**Figure 3.** IRF4 expression segregates between IL-10 high and IL-10 low Th1 cells. A, CD4 T cells were cultured under Th1 or Th2 conditions for 5 days before stimulation with anti-CD3. Supernatants were analyzed for IL-10 concentration using ELISA. B, IRF4 protein level was assessed by immunoblot in control (MIEG) and IRF4-transfected phoenix cells, as well as unstimulated and 6-h stimulated naive T cells, Th1, and Th2 cells. C, IRF4 protein level was analyzed in Th1 cells treated with monensin for the last 3 h of a 6-h stimulation with anti-CD3 before intracellular staining for IL-10 and IRF4. IRF4 expression in cells gated for high or low expression of IL-10. D, Differentiating Th1 cells were transduced with control (MIEG) or IRF4-expressing retrovirus as in Fig. 2B. Data in all panels are representative of two to three experiments.
addition of a condition where PU.1- and IRF4-expressing vectors were cotransfected. Results demonstrate that PU.1 also interferes with the IRF4-dependent induction of the Il4 and Il10 promoter reporter vectors (Fig. 5E). Overall, these results suggest that PU.1 interactions limit the ability of IRF4 to transactivate Th2 cytokines.

**IRF4 expression profile in subpopulations of Th2 cells**

The analysis in Fig. 1 is based on two-state separation of cells into IL-10 high and low cells. However, IL-10 high and low cells can be further divided based on coexpression of other cytokines. Because IRF4 induced production of IL-4 and IL-10, we examined the expression of IRF4 in populations of IL-4- and IL-10-positive cells by intracellular staining. IRF4 expression was highest in Th2 cells that were double-positive for IL-4 and IL-10, and lowest in cells that did not secrete either cytokine (Fig. 6). Interestingly, expression was intermediate but similar in IL-4- and IL-10-single positive cells. Results are shown for BALB/c cells and similar patterns are observed for C57BL/6 cells. Thus, while IRF4 promotes IL-4 and IL-10 production, other factors also contribute to the decision of a cell to make one or both cytokines.

**Discussion**

IRF4 plays an important role in the development of Th2 and Th17 cells (14). In *Irfa*−/− mice, development of Th2 cells is decreased, suggesting that it plays a role in the differentiation process (14). However, a role in differentiation does not preclude involvement in the regulation of specific cytokines in differentiated Th2 cells. In this report, we demonstrate that IRF4 contributes to the heterogeneity of Th2 populations by increasing production of IL-4 and IL-10 and decreasing expression of *Il9*, while having no effects on IL-5 or IL-13. IRF4 expression segregates in Th2 cells between IL-10 high and low cells, directly binds to and transactivates the *Il10* gene, and ectopic expression of IRF4 can increase IL-10 production from IL-10-low cells. Thus, IRF4 is an instructive factor in establishing Th2 heterogeneity.

IL-10 is a regulatory cytokine produced by a number of cells, including Th2 cells and *Il10* regulation in each cell type may be distinct. IL-10 plays a critical role in controlling inflammation in vivo by selectively suppressing the expression of proinflammatory cytokines. In this report, we show that IL-10 high and low cells have differing effects on cocultured cells in vitro, and it is likely that functions differ in vivo as well. In various cell types, the molecular mechanism regulating the expression of IL-10 involves binding of IRF1 and STAT3 to the promoter region of the *Il10* gene locus (27, 34), and the regulation of the level of IL-10 mRNA by Sp1 and Sp3 (35). In Th2 cells, GATA-3 remodels the *Il10* locus (34), and Jun family proteins bind the CNS3 region to induce transcription (26). Moreover, IL-10 production requires repeated stimulation to be completely imprinted within the Th2 population (36). In this report, we also show that IRF4 contributes to *Il10* expression in Th2 and Th1 cells. The IL-12-dependent production of IL-10 in Th1 cells has been documented in human and mouse cells (27, 37, 38). Clearly, additional factors contribute to

| Figure 4. | IRF4 binds to the IL-10 promoter and transactivates reporters with *Il10* regulatory regions. A, Representation of the mouse IL-10 gene with the arrow indicating the translational start and black boxes representing exons. The regions used for promoter and CNS3 reporters and ChIP are indicated. B, For DAPA analysis, Th2 nuclear extract was incubated with (+) or without (−) a biotinylated oligonucleotide corresponding to an IRF4 consensus binding site in the Il10 promoter, before precipitation with streptavidin-agarose and IRF4 immunoblot. Competitors (fold excess indicated) to demonstrate specificity of the Il10p oligonucleotide-IRF4 interaction included oligonucleotides containing an IRF4 binding site from the Igα gene, the Il10 promoter oligonucleotide containing a deletion of IRF4 binding site, and an oligonucleotide containing a GATA-3 consensus site. Competitors were incubated with Th2 extract for 15 min before incubation with the biotinylated oligonucleotide. Direct immunoblot of Th2 extract is indicated as Th2. C, ChIP assay of IRF4 binding to Il10, Il4, and Il9 promoter regions; *Il10* CNS3; and Il4Va. Quantification of IRF4 binding was performed using qPCR and is shown as percent input. To calculate percent input, ChIP results for the specific Ab were determined using a standard curve of input DNA from the same cells. Control IgG ChIP results are subtracted from specific Ab ChIP results and results are shown as the mean specific binding of replicates ± SD and are representative of three experiments. D, Analysis of luciferase assays from EL4 cells transfected with reporter vectors containing Il4 promoter, Il10 promoter, and CNS3 regulatory regions, or an NFAT reporter and either empty vector or an IRF4-expressing vector. Relative luciferase (normalized to EL4 cells transfected with empty vector) are shown in cells that were cultured in the presence (stimulated) or absence (unstimulated) of PMA plus ionomycin. Results are representative of two to three experiments. *, *p < 0.05.
the difference in IL-10 production by Th1 and Th2 cells, including that GATA-3 is not expressed in Th1 cells, c-jun and JunB bind to the \textit{Il10} CNS3 in Th2 but not Th1 cells (26), and Ets-1 is a negative regulator of IL-10 production in Th1 cells (39). We demonstrate that in Th2 cells, IRF4 binds directly to the \textit{Il10} locus and is able to transactivate \textit{Il10} regulatory element reporter plasmids. Our results parallel the recent description of a role for IRF4 in Treg cells where \textit{Il10} was one of the prominently regulated genes (15).

The fact that IRF4 binding sites exist in the \textit{Il10} promoter and CNS3 regions, and that it binds to the same regulatory element as Jun containing complexes suggests that these factors may cooperate. We did not observe strong interactions of IRF4 with GATA-3, suggesting that while these factors both contribute to \textit{Il10} expression, direct interactions are not required.

There are interactions of IRF4 with PU.1, which we previously demonstrated decreased IL-10 production (8). In the absence of PU.1, IRF4 had greater potential to bind \textit{Il10}, transactivate the \textit{Il10} promoter, and increase IL-10 production, suggesting that, at least in part, PU.1 is able to modulate \textit{Il10} through the ability to interfere with IRF4 activity. It was surprising that we did not see increased IL-10 in \textit{Sfpilck}\textsuperscript{+/−−} CD4 T cells cultured under Th2 conditions and transduced with control or IRF4-expressing retroviruses as described in Fig. 3B. IL-10 levels were determined by ELISA. E. Analysis of luciferase assays from EL4 cells transfected with reporter vectors containing \textit{Il10} promoter or \textit{Il4} promoter, and either empty vector, IRF4-expressing vector, or IRF4- and PU.1-expressing vectors. Relative luciferase (normalized to EL4 cells transfected with empty vector) are shown in cells that were cultured in the presence of PMA plus ionomycin. Results are representative of two to three experiments. *, p < 0.05.

FIGURE 5. PU.1 interacts with and limits the activity of IRF4. A, Nuclear protein (1 mg) from Th2 cells was incubated overnight with Abs to PU.1, IRF4, or GATA-3 (IP) or control Ab (Ab). Immunocomplexes were precipitated with protein G beads. Immunoblot (IB) was performed for PU.1, IRF4, and GATA-3 in each of the precipitates. B, RNA was isolated from IL-10 low and IL-10 high cells sorted as in Fig. 1A and analyzed using qPCR for \textit{Sfpil} expression. C, ChIP assay for IRF4 binding to \textit{Il10} promoter and CNS3 regions, \textit{Il10} CNS3 regions was performed as described in Fig. 4B with chromatin from WT and \textit{Sfpilck}\textsuperscript{+/−−} CD4 T cells cultured under Th2 conditions. IRF4 ChIP was performed as in Fig. 4C. Results are shown with control IgG ChIP subtracted and indicate mean ± SD of replicate samples that are representative of three experiments. D, WT and \textit{Sfpilck}\textsuperscript{+/−−} CD4 T cells were cultured under Th2 conditions and transduced with control or IRF4-expressing retroviruses as described in Fig. 3B. IL-10 levels were determined by ELISA. E, Analysis of luciferase assays from EL4 cells transfected with reporter vectors containing \textit{Il10} promoter or \textit{Il4} promoter, and either empty vector, IRF4-expressing vector, or IRF4- and PU.1-expressing vectors. Relative luciferase (normalized to EL4 cells transfected with empty vector) are shown in cells that were cultured in the presence of PMA plus ionomycin. Results are representative of two to three experiments. *, p < 0.05.

FIGURE 6. IRF4 heterogeneity in Th2 cells. CD4 T cells were cultured under Th2 conditions and IRF4 protein level was analyzed in cells treated with monensin for the last 3 h of a 6-h stimulation with anti-CD3 before intracellular staining for IL-4, IL-10, and IRF4. The mean fluorescence intensity (MFI) of IRF4 staining is shown in the table for each of the gated populations.

<table>
<thead>
<tr>
<th>Condition</th>
<th>MFI</th>
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<tbody>
<tr>
<td>IL-10-</td>
<td>1538</td>
</tr>
<tr>
<td>IL-10-</td>
<td>658</td>
</tr>
<tr>
<td>IL-10+</td>
<td>3768</td>
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<tr>
<td>IL-4+</td>
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The difference in IL-10 production by Th1 and Th2 cells, including that GATA-3 is not expressed in Th1 cells, c-jun and JunB bind to the \textit{Il10} CNS3 in Th2 but not Th1 cells (26), and Ets-1 is a negative regulator of IL-10 production in Th1 cells (39). We demonstrate that in Th2 cells, IRF4 binds directly to the \textit{Il10} locus and is able to transactivate \textit{Il10} regulatory element reporter plasmids. Our results parallel the recent description of a role for IRF4 in Treg cells where \textit{Il10} was one of the prominently regulated genes (15). The fact that IRF4 binding sites exist in the \textit{Il10} promoter and CNS3 regions, and that it binds to the same regulatory element as Jun containing complexes suggests that these factors may cooperate. We did not observe strong interactions of IRF4 with GATA-3, suggesting that while these factors both contribute to \textit{Il10} expression, direct interactions are not required.

There are interactions of IRF4 with PU.1, which we previously demonstrated decreased IL-10 production (8). In the absence of PU.1, IRF4 had greater potential to bind \textit{Il10}, transactivate the \textit{Il10} promoter, and increase IL-10 production, suggesting that, at least in part, PU.1 is able to modulate \textit{Il10} through the ability to interfere with IRF4 activity. It was surprising that we did not see increased IL-10 in \textit{Sfpilck}\textsuperscript{+/−−} control-transduced cells (Fig. 5D), and this may be a result of the culture conditions required for retroviral transduction. The effects of PU.1 deficiency resulting in increased Th2 cytokine production are most dramatic when TCR stimulation is limiting (H. C. Chang, S. L. Nutt, and M. H. Kaplan, submitted for publication). However, transduction of cells with limiting TCR stimulation was not efficient, and as a result of using optimal stimulation conditions for the retroviral transduction, we did not observe altered IL-10 production in the absence of IRF4 transduction. The role of PU.1 must also be placed in the context of interactions with multiple transcription factors (Fig. 5A) and with the heterogeneity of the Th2 population where PU.1 is...
differentially expressed in subpopulations of cells. As previously noted, PU.1 is expressed highly in IL-4 low cells, and in this report is also expressed in IL-10-high cells. This might suggest that PU.1 is most highly expressed in an IL-4 low/IL-10 high population. However, it has thus far been difficult to sort cells stained for two cytokines preventing a more thorough analysis of this issue. As PU.1 expression is only present in a subpopulation of Th2, the effects of deficiency on IRF4 function might be obscured in a bulk Th2 population, unless IRF4 is overexpressed (Fig. 5D). Moreover, as ectopic PU.1 expression decreased IL-5 and IL-13 production, as well as IL-4 and IL-10, it is clear that interactions with IRF4 only account for a portion of the observed function of PU.1 in Th2 cells.

The reciprocal regulation of II10 and II9 in Th2 cells is striking and distinct from the IL-9 and IL-10-producing cells present in cultures primed with TGFβ and IL-4 (40, 41). Although transduction of IRF4 in Th2 cells decreases II9 expression, we observed only minimal IRF4 binding to the II9 promoter in a ChIP assay. This suggested that the effects of IRF4 could be indirect, through the induction of IL-10. Indeed, neutralizing IL-10 in IL-10-high cells, that express low levels of II9, modestly increased II9 mRNA (data not shown). However, it is not clear which IL-10 activated pathways might be responsible for this regulation. It is also not clear why a cell would be specialized to express only one of these cytokines. IL-9 is a pleiotropic cytokine involved in the pathologic and physiologic evolution of asthma by recruiting eosinophils and lymphocytes to the lung, inducing mucus hypersecretion, mast cells hyperplasia in concert with IL-4, IL-5, and IL-13 (42), while IL-10 is a suppressive cytokine that may modulate many of these processes. It is possible that secretion of IL-9 by Th2 cells would only be effective if target cells did not receive a conflicting signal generated by IL-10. In this manner, Th2 heterogeneity may reflect functional specialization of cell types within the inflammatory microenvironment.

Increasing evidence suggests that the establishment of Th2 heterogeneity is not stochastic, but rather instructive, based on the expression of specific factors. As such, a growing list of transcription factors has specific effects on individual Th2 cytokines. IRF4 is induced following T cell activation, and expression is further increased following Th2 differentiation. Importantly, the level of IRF4 in IL-10 low cells is increased compared with that in recently activated T cells. As we have shown, IRF4 activates IL-10 and IL-4 production, while decreasing IL-9 and having little effect on IL-5 or IL-9 expression in Th2 cells (43). Similarly, Pias1 increases IL-13 production without affecting IL-4 or IL-5 expression (44). We have shown that PU.1 decreases expression of many Th2 cytokines, but increases expression of CCL22, a chemokine associated with Th2 inflammation (8). BOB.1/0BF.1 regulates PU.1 expression in Th2 cells and also affects the potential for Th2 cytokine production (45). Moreover, the expression levels of each of these factors, and other factors that contribute to Th2 cytokine production exist in gradients that correlate with cytokine producing phenotypes (Fig. 6). The similar level of expression of IRF4 in both IL-4 and IL-10 single-positive cells supports the idea that other positive- or negative-acting factors overlay on the IRF4 gradient to generate the specific patterns of cytokine secretion. The mosaic of transcription factor gradients ultimately results in the heterogeneity observed in cytokine production from individual cells.

In this report, we have identified IRF4 as a regulator of Th2 heterogeneity by enhancing or decreasing the production of specific cytokines. IRF4 function, like GATA-3 as described in our previous report (8), is limited by the expression of PU.1 in Th2 cells, which binds IRF4 and decreases binding to target genes including II10. Future work will examine how these factors interact to generate the population phenotype and what signals determine the expression of each factor within individual cells.

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References


