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The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma

Ryoji Yagi[1], **Ilkka S. Junttila**[1], **Gang Wei**[2], **Joseph F. Urban Jr**[3], **Keji Zhao**[2], **William E. Paul**[1], and **Jinfang Zhu**[1,*]

[1] Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

[2] Laboratory of Molecular Immunology, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

[3] Diet, Genomics, and Immunology Laboratory, Beltsville Human Nutrition Research Center, U.S. Department of Agriculture, Beltsville, Maryland, 20705, USA

SUMMARY

The transcription factor GATA3 is crucial for the differentiation of naïve $CD4^+$ T cells into T helper 2 (Th2) cells. Here we show that deletion of *Gata3* allowed the appearance of interferon γ (IFNγ)-producing cells in the absence of interleukin-12 (IL-12) and IFNγ. Such IFNγ production was transcription factor T-bet-independent. Another T-box-containing transcription factor Eomes, but not T-bet, was induced both in GATA3-deficient $CD4^+$ T cells differentiated under Th2 cell conditions, and in Th2 cells with enforced Runx3 expression, contributing to IFNγ production. GATA3 over-expression blocked Runx3-mediated Eomes induction and IFNγ production, and GATA3 protein physically interacted with Runx3 protein. Furthermore, we found that Runx3 directly bound to multiple regulatory elements of the *Ifng* gene, and that blocking Runx3 function in either Th1 or GATA3-deficient "Th2" cells results in diminished IFNγ production by these cells. Thus, the Runx3-mediated pathway, actively suppressed by GATA3, induces IFNγ production in a STAT4 and T-bet-independent manner.

INTRODUCTION

Naïve $CD4^+$ T cells differentiate into at least four types of T helper (Th) cells, including Th1, Th2 cells, inducible T regulatory cells and Th17 cells. Th1 cells produce cytokines such as IFNγ and lymphotoxin alpha and activate macrophages and $CD8^+$ T cells to induce immunity against intracellular pathogens, whereas Th2 cells produce signature cytokines, interleukin-4 (IL-4), IL-5, IL-9 and IL-13, that are involved in host defense against extracellular pathogens such as helminths (Ansel et al., 2006; Murphy and Reiner, 2002; Zhu and Paul, 2008). Differentiation fate is determined by several factors, including the nature and dose of antigen, the type of co-stimulation, and the cytokine milieu.

Both IFNγ and IL-12 play important roles in Th1 differentiation. The capacity of T cells to produce IFNγ is programmed by various transcription factors including STAT4, two T-box

*Correspondence: J. F. Z. (jfzhu@niaid.nih.gov). Phone: 301-402-6662, FAX: 301-480-7352.

protein family members, T-bet and Eomesodermin (Eomes), and Runx3. STAT4 is activated by IL-12; STAT4-deficient $CD4^+$ T cells have a defect in IFNγ production (Jacobson et al., 1995; Kaplan et al., 1996; Thierfelder et al., 1996; Watford et al., 2004). T-bet is induced mainly through an IFNγ-STAT1-dependent pathway (Afkarian et al., 2002; Lighvani et al., 2001), but the IL-12-STAT4 pathway also contributes to T-bet up-regulation (Yang et al., 2007). T-bet not only promotes Th1 cell differentiation, but also represses Th2 cell differentiation by suppressing GATA3 expression (Usui et al., 2006) and reducing the binding of GATA3 to DNA (Hwang et al., 2005; Szabo et al., 2000). T-bet deficient (*Tbx21*$^{-/-}$) "Th1" cells and *Tbx21*$^{-/-}$ NK cells produce less IFNγ than do wild type (WT) cells. Although T-bet deficiency does not affect IFNγ production by $CD8^+$ T cells *in vitro* (Szabo et al., 2002), it results in reduced numbers of IFNγ-producing antigen-specific $CD8^+$ T cells in response to LCMV infection (Intlekofer et al., 2007; Joshi et al., 2007). Pearce *et al.* reported that IFNγ production by *Tbx21*$^{-/-}$ $CD8^+$ T cells *in vitro* was dependent on the expression of Eomes (Pearce et al., 2003).

Runx3, a critical transcription factor for silencing CD4 expression during T cell development (Taniuchi et al., 2002), has been reported to be expressed at higher amount in Th1 cells than in Th2 cells (Djuretic et al., 2007; Naoe et al., 2007). Runx3 enhances IFNγ production although the detailed mechanism through which it does so is not clear (Djuretic et al., 2007). In addition, Runx3 has been reported to directly repress IL-4 transcription by binding, in collaboration with T-bet, to the DNase I hypersensitivity (HS) IV region of the *Il4* gene (Djuretic et al., 2007). *Runx3*$^{fl/fl}$-CD4-Cre mice spontaneously show an asthma-like phenotype, including increased serum IgE and infiltration of lymphoid cells in the lung and bronchoaveolar lavage fluid, implying that Runx3 also is important for repressing Th2 responses (Naoe et al., 2007).

IL-4 and IL-2 are indispensable for *in vitro* Th2 differentiation (Cote-Sierra et al., 2004; Yamane et al., 2005; Zhu et al., 2003). The regulation of Th2 differentiation and of the capacity of these cells to produce Th2 cytokines depends on several transcription factors including STAT5, STAT6 and GATA3 (Zhu et al., 2006). GATA3, the "master" transcription factor for Th2 differentiation, is up-regulated both by TCR stimulation and IL-4-STAT6 signaling (Ouyang et al., 1998; Zheng and Flavell, 1997). By contrast, GATA3 expression is diminished during Th1 differentiation. Enforced GATA3 expression in developing Th1 cells induces IL-4-producing capacity. The importance of GATA3 expression during Th2 differentiation, both *in vitro* and *in vivo*, has been confirmed utilizing GATA3-conditionally-deficient mice (Pai et al., 2004; Zhu et al., 2004). These experiments showed that GATA3 is critical for promoting Th2 cell expansion as well as for Th2 cell differentiation.

GATA3 also negatively regulates Th1 differentiation. It represses IFNγ production through an IL-4-independent pathway (Ouyang et al., 1998; Usui et al., 2003). Ouyang *et al.* showed that over-expression of GATA3 in Th1 cells inhibited IL-12Rβ2 expression, which is normally induced under Th1 conditions (Ouyang et al., 1998). However, enforced IL-12Rβ2 expression in GATA3-over-expressing Th1 cells does not restore IFNγ production implying that another mechanism, possibly down-regulation of STAT4, contributes to GATA3 repression of Th1 differentiation (Usui et al., 2003).

Interestingly, GATA3-deficient $CD4^+$ T cells cultured under Th2 conditions produced IFNγ, indicating that endogenous GATA3 is required to actively repress IFNγ production in Th2 cells and that without GATA3, IFNγ production can be induced in the absence of the two established Th1-inducing factors, IL-12 and IFNγ (Pai et al., 2004; Zhu et al., 2004). Furthermore, in GATA3-conditionally-deficient mice, $CD4^+$ T cells produced IFNγ in response to *Nippostrongylus brasiliensis* infection, an infection that elicits strong Th2 responses in WT mice. We were intrigued by this finding and became interested in understanding how GATA3 mediated IFNγ repression. Here, we show that GATA3 suppresses both STAT4 and T-bet-

independent Eomes expression and IFNγ production. Our results indicate that the ratio of Runx3 to GATA3 determines the degree of IFNγ expression. This "balanced" regulation may be explained by the capacity of GATA3 and Runx3 to bind to each other. Furthermore, we show that Runx3 binds to many critical regulatory elements of *Ifng* gene, some of which co-localize with T-bet binding sites, suggesting that Runx3 directly regulates IFNγ production.

RESULTS

Deletion of *Gata3* Allows IFNγ Production Independently of IL-12-STAT4 and IFNγ-T-bet

GATA3 is important for $CD4^+$ T cell development in the thymus and for Th2 cell differentiation in the periphery (Ho et al., 2009). To investigate the role of GATA3 in T cells at different developmental stages, we generated mice in which exon4 of *Gata3* is flanked by two loxP sites (*Gata3*$^{fl/fl}$). *Gata3* deletion by CD4-Cre greatly diminished the development of $CD4^+$ T cells. Of the few peripheral $CD4^+$ T cells that do appear in such mice, the majority displayed a memory phenotype (Zhu et al., 2004). We also reported that deletion of *Gata3* by OX40-Cre in activated T cells abolished Th2 responses to *Nippostrongylus brasiliensis* infection and allowed the production of IFNγ by cells from infected mice. However, the effect of *Gata3* deletion in naïve $CD4^+$ T cells has not been previously tested. The expression of humanized Cre driven by the distal Lck promoter (dLck-Cre Tg, line 3779) efficiently deletes floxed genes in CD4 or CD8 single positive T cells in the thymus and naïve $CD4^+$ T cells in the periphery (Zhang et al., 2005). To investigate the function of GATA3 in naïve $CD4^+$ T cells, *Gata3*$^{fl/fl}$-dLck-Cre mice were prepared by crossing *Gata3*$^{fl/fl}$ mice to dLck-Cre Tg mice.

$CD4^+$ and $CD8^+$ T cells developed normally in the thymi of *Gata3*$^{fl/fl}$-dLck-Cre mice and the majority of $CD4^+$ and $CD8^+$ T cells in periphery of these mice displayed a naïve phenotype ($CD62L^{hi}CD44^{lo}$), in a percentage that was indistinguishable from that of control mice (Figure S1A). The deletion efficiency of the *Gata3* gene in $CD4^+$ and $CD8^+$ T cells from *Gata3*$^{fl/fl}$-dLck-Cre mice was approximately 70% and 90%, respectively (Figure S1B). Thus, unlike *Gata3*$^{fl/fl}$-CD4-Cre mice in which GATA3-deficient naïve $CD4^+$ T cells were not present in substantial numbers because the gene was deleted too early during T cell development, *Gata3*$^{fl/fl}$-dLck-Cre mice provide a unique opportunity to study the role of GATA3 at early stages of T cell differentiation from naïve $CD4^+$ T cells.

Naïve $CD4^+$ T cells were isolated from *Gata3*$^{fl/fl}$-dLck-Cre or *Gata3*$^{fl/fl}$ mice and then cultured with soluble anti-CD3 and anti-CD28 in the presence of T-depleted splenocytes under Th1 (IL-12 and anti-IL-4) or Th2 conditions (anti-IL-12, anti-IFNγ and IL-4). *Gata3* deletion in Th1 cells did not affect IFNγ production by these cells (Figure 1A, left panel). As expected, IL-4 production by these *Gata3*$^{fl/fl}$-dLck-Cre "Th2" cells upon stimulation was dramatically lower than that IL-4 production by controls (31.2% versus 69.9%, Figure 1A, middle panel). IFNγ production was induced in ~24% of the "Th2" cells from *Gata3*$^{fl/fl}$-dLck-Cre mice even though both IL-12 and IFNγ were neutralized in the culture. Because the deletion of *Gata3* by dLck-Cre is incomplete, intracellular staining of GATA3 was carried out to identify the *Gata3*-deleted cells (Figure 1A, right panel). Among the *Gata3*$^{fl/fl}$-dLck-Cre "Th2" cells, IL-4 was produced only by $CD4^+$ T cells from which GATA3 had not been deleted whereas IFNγ was produced mainly by $CD4^+$ T cells that had deleted the gene. A substantial amount of IFNγ was detected by ELISA in the supernatant of *Gata3*$^{fl/fl}$-dLck-Cre "Th2" cells (Figure 1B). Careful kinetic study showed that IFNγ expression by *Gata3*$^{fl/fl}$-dLck-Cre cells cultured under Th2 conditions, similar to that by WT Th1 cells, was most prominent at 48hr after stimulation (data not shown). These data imply that GATA3 is crucial for the induction of IL-4 production and that loss of GATA3 in naïve $CD4^+$ T cells allows IFNγ production when they become activated in the absence of engagement of either the IL-12-STAT4 pathway or the IFNγ-STAT1 pathway.

T-bet is the "master regulator" of IFNγ production in Th1 cells (Szabo et al., 2000). To test whether T-bet is involved in the IFNγ production in GATA3-deficient "Th2" cells, we generated *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ mice deficient in both GATA3 and T-bet. Whereas ~15% of *Tbx21*$^{-/-}$ Th1 cells produced IFNγ, ~67% of *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ Th1 cells did so (Figure 1C, left panel). Identical to the data shown in Figure 1A, more than 90% of *Gata3*$^{fl/fl}$ and *Gata3*$^{fl/fl}$-dLck-Cre Th1 cells in this experiment produced IFNγ (data not shown). Importantly, ~25% of *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ cells cultured under Th2 or neutral conditions (without addition of exogenous cytokines and antibodies to cytokines) produced IFNγ (Figure 1C, middle and right panel). *Tbx21*$^{-/-}$ and *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ were also infected with *Nippostrongylus brasiliensis*. IL-4 and IFNγ mRNA were measured by quantitative PCR on day 9 after infection (Figure 1D). IL-4 expression was dramatically reduced and IFNγ expression was enhanced in *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ cells compared to *Tbx21*$^{-/-}$ cells. These data indicate that GATA3 regulates T-bet-independent IFNγ production both *in vitro* and *in vivo*.

Eomes but not T-bet is Up-regulated in *Gata3*$^{fl/fl}$-dLck-Cre "Th2" Cells

To determine how deletion of GATA3 allowed IL-12-IFNγ-independent IFNγ production in CD4$^+$ T cells, the expression of the IFNγ-inducing transcriptional factors T-bet, Eomes, Runx3 and STAT4 was assessed by quantitative PCR in both WT and GATA3-deficient "Th2" cells (Figure 2A). Strikingly, Eomes, but not T-bet, mRNA was increased in GATA3-deficient "Th2" cells. Intracellular staining of Eomes and T-bet confirmed that GATA3-deficient "Th2" cells expressed Eomes but not T-bet (Figure 2B). Virtually all the cells that had deleted GATA3 from *Gata3*$^{fl/fl}$-dLck-Cre "Th2" group expressed Eomes, although only a small proportion of WT Th1 cells expressed this transcription factor. Furthermore, GATA3-deficient Th1 cells failed to express Eomes, suggesting that either Th1 factors inhibit Eomes expression or IL-4 induces Eomes when GATA3 is absent. STAT4 was also up-regulated in *Gata3*$^{fl/fl}$-dLck-Cre "Th2" cells at both mRNA (Figure 2A) and protein amounts (Figure 2C) consistent with the previous report that GATA3 suppresses STAT4 expression in Th2 cells (Usui et al., 2003). Runx3 was slightly increased at mRNA levels and protein levels when *Gata3* was deleted from differentiating Th2 cells, however, such Runx3 expression is still much lower than its expression in Th1 cells. Thus, the loss of GATA3 during Th2 differentiation results in increased expression of Eomes and STAT4 but not T-bet.

Th1 cells also preferentially express IL-12Rβ2 and CXCR3. In GATA3-deficient "Th2" cells, the expression of IL-12Rβ2 was slightly enhanced, while CXCR3 expression was dramatically up-regulated (Figure S2). Up-regulation of STAT4 expression and a slight increase of IL-12Rβ2 could contribute to IFNγ production if IL-12 were present; however, IL-12 is not available in the Th2 cultures and we failed to detect STAT4 phosphorylation in *Gata3*$^{fl/fl}$-dLck-Cre cells cultured under Th2 conditions (data not shown). Therefore, the up-regulation of Eomes is most likely responsible for IFNγ production in GATA3-deficient "Th2" cells.

Eomes but not T-bet Contributes to the IFNγ Production in GATA3-deficient "Th2" Cells

Eomes has been reported to play an important role in IFNγ production in CD8$^+$ T cells (Pearce et al., 2003). In addition, Eomes expression in CD4$^+$ T cells may contribute to optimal IFNγ production by these cells (Suto et al., 2006). To further address whether Eomes is capable of inducing IFNγ in Th2 cells, WT or *Tbx21*$^{-/-}$ CD4$^+$ T cells cultured under Th2 conditions were infected with an Eomes-GFP-Retrovirus (RV). 30.8% of WT and 23.5% of *Tbx21*$^{-/-}$ Th2 cells that had been infected with the Eomes-GFP-RV produced IFNγ (Figure 3A) whereas less than 1% of control-RV infected Th2 cells produced IFNγ. RV-driven Eomes expression was comparable to endogenous Eomes expression in GATA3-deficient "Th2" cells (Figure S3), suggesting Eomes in GATA3-deficient "Th2" cells is sufficient to induce IFNγ production

independently of T-bet. Interestingly, enforced Eomes expression in Th2 cells did not suppress IL-4 production.

To compare the functions of Eomes and T-bet, expression of mRNA of multiple genes in sorted Th2 cells that had been infected with T-bet- or Eomes-RV was measured by quantitative PCR. Eomes was less potent than T-bet in inducing IFNγ and IL-12Rβ2 and in suppressing GATA3 expression, however, it induced CXCR3 to a similar degree as did T-bet (Figure 3B). Both T-bet and Eomes are the members of T-box family and they bind to similar DNA sequences. A dominant negative form of T-bet (T-bet DN) has been reported to suppress the activities of both T-bet and Eomes (Mullen et al., 2002). Infection of "Th2" cells from both *Gata3*$^{fl/fl}$-dLck-Cre and *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ mice with T-bet DN-GFP-RV reduced the percentage of IFNγ-producing cells by a comparable degree, from 55% to 27% in the former and from 51% to 22% in the latter (Figure 3C), indicating that Eomes is at least partially responsible for the capacity of cells lacking GATA3 to produce IFNγ. It also suggests that a non-T-box family member may be responsible for a portion of the IFNγ production.

Runx3 Induces IFNγ Production and Eomes Expression

Runx3 is expressed in Th1 cells and is known to induce the capacity of these cells to produce IFNγ as well as to suppress their production of IL-4 (Djuretic et al., 2007; Naoe et al., 2007). Because both Runx3 and Eomes are highly expressed in CD8$^+$ T cells (Egawa et al., 2007; Pearce et al., 2003), we also tested the involvement of Runx3 in IFNγ production by Th2 cells. Runx3-GFP-RV transduction into WT Th2 cells resulted in 41.4% of the cells being able to secrete IFNγ (Figure 4A, upper panel). The acquisition of IFNγ-producing capacity by over-expression of Runx3 is independent of T-bet since *Tbx21*$^{-/-}$ Th2 cells that had been infected with the Runx3-GFP-RV were similar to WT Runx3-GFP-RV-infected cells in their ability to produce IFNγ (Figure 4A lower panel). Unlike enforced Eomes expression, Runx3 expression in Th2 cells dramatically reduced the frequency of IL-4-producing cells, from 26% to 3.5% in WT and from 21% to 3.8% in *Tbx21*$^{-/-}$ Th2 cells. Thus, the suppression of IL-4-producing capacity by Runx3 is also T-bet-independent.

Interestingly, enforced Runx3 expression strongly induced Eomes mRNA with minimal effects on the expression of T-bet (Figure 4B) and GATA3 (data not shown). IFNγ production induced by enforced Runx3 was again partially blocked by T-bet DN (Figure 4C), suggesting that Runx3 induces IFNγ production partly through its up-regulation of Eomes expression.

Runx3 and T-bet Bind to Multiple Regulatory Elements of the *Ifng* Gene

To test whether Runx3 is involved in IFNγ production in Th1 cells, we constructed a dominant negative Runx3 (Runx3 DN) based on a previous report (Hayashi et al., 2000). Infecting normal Th1 cells with Runx3 DN-RV, similar to that with T-bet DN-RV, dramatically reduced the percentage as well as the mean fluorescence intensity (MFI) of the IFNγ-producing cells (Figure 5A), suggesting optimal IFNγ production in normal Th1 cells requires T-bet as well as Runx3 function.

To explore the molecular mechanisms underlying these effects, we performed ChIPseq (chromatin immunoprecipitation in combination with high throughput deep sequencing) to identify the binding sites of Runx3 and T-bet in Th1 cells. An antibody to CBFβ, which is a co-factor of Runx complexes, was used since we failed to identify ChIP quality Runx3 antibody.

The data showed that Runx protein complex weakly binds to many sites within the *Il4–Il13* locus including DNase I hypersensitivity (HS) IV (Figure S4A), the site to which Runx has been previously reported to bind. By contrast, T-bet failed to bind to HS IV, consistent with

our data that Runx3-mediated suppression of IL-4 production does not require T-bet. No apparent binding clusters for Runx protein complex can be identified in the vicinity of the *Eomes* gene (Figure S4B) although there are clusters of CBFβ binding sites at the *Eomes* neighbor gene *Azi2* (data not shown), suggesting Runx3 may regulate Eomes expression through a long-distance interaction if such action is direct or Runx3 indirectly regulates Eomes.

Importantly, both Runx and T-bet strongly bind to many regulatory elements of the *Ifng* gene (Figure 5B). The common binding sites for these two transcription factors are at conserved non-coding sequence (CNS) -34, CNS-22, *Ifng* promoter (HSI) and CNS+29. In addition, Runx3 uniquely binds to CNS-6, HSII, HSIII and CNS+55 whereas T-bet uniquely binds to CNS+18–20 and CNS+46. These data suggest both Runx3 and T-bet regulate IFNγ production by binding to many critical regulatory elements of the *Ifng* gene.

GATA3 Represses the Capacity of Runx3 to Induce IFNγ Expression

We have shown here that loss of GATA3 expression in Th2 cells resulted in the acquisition of IFNγ-producing capacity that was correlated with Eomes, but not T-bet expression, and that Runx3 over-expression in Th2 cells resulted in IFNγ-producing capacity and up-regulated Eomes. However, the relationship between GATA3 and Runx3 in regulating Eomes and IFNγ expression remains unknown.

We first asked whether it appeared that GATA3 and Runx3 acted in a balanced manner. Th2 cells were co-infected with a Runx3-Thy1.1-RV and a GATA3-GFP-RV. While cells that were infected with neither virus could produce little IFNγ, a substantial proportion of the cells infected with Runx3-RV alone produced IFNγ (23.5%) but this was reversed in cells that had been infected with both the Runx3 and GATA3-RVs (3.5%, Figure 6A). The amount of IFNγ produced by stimulated doubly-RV-infected cells separated based on the relative intensities of GFP and Thy1.1 expression, representing the relative expression of GATA3 and Runx3, respectively, correlated with the ratio of Runx3 and GATA3 expression (Figure 6B). By contrast, an excess of GATA3 did not prevent Runx3 from suppressing IL-4 production. Consistent with its role in regulating IFNγ production, Eomes expression was only enhanced in the cells expressing retroviral Runx3 alone; such induction was repressed by additional GATA3 expression (Figure 6C). These results imply that GATA3 suppresses Runx3-mediated Eomes induction and IFNγ production in a quantitative manner.

A possible explanation for such quantitative regulation would be that GATA3 could interact with Runx3 and thus block its ability to induce Eomes and IFNγ. Anti-GATA3 immunoprecipitated Runx3 from extracts of primary Th1 cells indicating an interaction between these two factors (Figure 7A). To determine which domains of GATA3 interact with Runx3, we prepared a series of GATA3 mutants (Figure S5A). HEK 293T cells were singly- or co-transfected with expression vectors encoding Runx3 and WT or mutant GATA3. Deletion of the N-terminus or Zinc-fingers of GATA3 abolished its binding to Runx3 whereas a C-terminal deleted GATA3 retained its ability to bind to Runx3 (Figure 7B). The GATA3 mutants that failed to interact with Runx3 also lost their capacity to suppress Runx3-mediated IFNγ production in Th2 cells (Figure 7C). However, although the GATA3 C-terminal deletion mutant was fully capable of suppressing Runx3-mediated IFNγ production (Figure 7C), consistent with its ability to bind to Runx3, it had a substantial reduction in its ability to induce IL-4 in Th1 cells (Figure S5B).

To confirm that the IFNγ production by GATA3-deficient “Th2” cells is Runx3-dependent but T-bet-independent, we generated triple deficient mice, *Gata3*$^{fl/fl}$ *Runx3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ (Figure 7D). Eomes expression was only slightly reduced in triple-deficient mice suggesting that there is a Runx3-independent mechanism for Eomes induction when GATA3 is absent in Th2 cells. However, despite continued expression of Eomes, the proportion of

IFNγ-producing cells was dramatically reduced in *Gata3*$^{fl/fl}$ *Runx3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ "Th2" cells compared to *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ (~8% versus ~32%) suggesting that Runx3 is required for the bulk of T-bet-independent IFNγ production in GATA3-deficient "Th2" cells and that it is the balance between GATA3 and Runx3 that controls IFNγ production.

DISCUSSION

GATA3 is a crucial transcription factor in the regulation of T helper cell differentiation. Not only is GATA3 critical for inducing Th2 differentiation, but it is also essential for repressing Th1 differentiation. It has been reported that GATA3 represses the IL-12-STAT4-IFNγ pathway during Th2 differentiation through multiple mechanisms including the suppression of both IL-12Rβ2 and STAT4 (Ouyang et al., 1998; Usui et al., 2003). Here, by using a mouse line carrying a Cre transgene that conditionally deletes *Gata3* in naïve CD4$^+$ T cells, we demonstrated that GATA3 actively suppresses IFNγ production even in the presence of IL-4, anti-IL-12 and anti-IFNγ. Without GATA3, the expression of STAT4 is increased, but only a modest increase of IL-12Rβ2 mRNA expression was noted. IL-12Rβ2 expression can be regulated by the IL-12-STAT4 and the IFNγ-T-bet pathways (Afkarian et al., 2002; Lawless et al., 2000; Ouyang et al., 1998). Thus, during Th1 differentiation in the presence of IL-12, the direct target suppressed by enforced GATA3 is likely to be STAT4 and through regulating the IL-12-STAT4 pathway, GATA3 indirectly affects IL-12Rβ2, IFNγ, and T-bet expression.

In this report, we describe Runx3-Eomes-mediated IFNγ production in GATA3-deficient "Th2" cells. Such IFNγ production is IL-12-STAT4- and IFNγ-T-bet-independent. The expression of Eomes, but not T-bet, is enhanced in GATA3-deficient CD4$^+$ T cells cultured under Th2 conditions, where both IL-12 and IFNγ are neutralized. Enforced Eomes expression during Th2 differentiation induces the capacity of these cells to produce IFNγ in the absence of T-bet. Moreover, GATA3-single deficient and GATA3-T-bet-double deficient CD4$^+$ T cells cultured under Th2 conditions produce similar amount of IFNγ. Such IFNγ production is inhibited by T-bet DN, which has been reported to suppress the function of both T-bet and Eomes (Pearce et al., 2003). Although the inhibition of IFNγ production by T-bet DN is only partial, the data clearly imply that Eomes, not T-bet, contributes to IFNγ production in GATA3-deficient "Th2" cells. We have reported earlier that in *Gata3*-deleted "Th2" clones, T-bet expression was elevated (Zhu et al., 2006). Since these clones had been cultured for a long period of time, it is possible that the large amount of IFNγ produced by these clones in the culture, were not completely neutralized and that IFNγ was responsible for T-bet induction.

Expressing Runx3 in activated CD4$^+$ T cells cultured under Th2 conditions strongly suppresses IL-4 production. Although others have argued that the suppression of IL-4 by Runx3 requires T-bet (Djuretic et al., 2007), our results clearly demonstrate the suppression of IL-4 by Runx3 is as efficient in T-bet-deficient Th2 cells as in WT cells. In addition, the ChIPseq data show that the Runx protein complex but not T-bet binds to the HS IV site of the *Il4* locus in Th1 cells. However, T-bet but not Runx3 suppresses GATA3 expression. Thus, Runx3 and T-bet can collaborate in repressing IL-4 production through two different mechanisms--Runx3 directly suppresses IL-4 and T-bet indirectly affects IL-4 by repressing GATA3.

Besides suppressing IL-4 production, Runx3 induces IFNγ production in Th2 cells correlated with strong induction of Eomes, but not T-bet. Although there is only a slight increase of Runx3 expression when GATA3 is absent, deletion of Runx3 from GATA3-T-bet double deficient cells results in dramatic decrease in IFNγ production indicating that Runx3 is needed for IFNγ production in these cells. However, deletion of Runx3 from GATA3-T-bet double deficient cells only causes a modest decrease in Eomes expression suggesting Runx3 is redundant for Eomes induction in GATA3-deficient "Th2" cells. Furthermore, although Runx3 is highly expressed in Th1 cells, only a small percentage of these cells express Eomes even

when GATA3 is deleted, suggesting that Runx3 is not sufficient to induce Eomes expression in a Th1 environment. Whether the Eomes-expressing $CD4^+$ T cells represent a unique lineage of Th cells needs to be further studied. Nevertheless, it has been recently reported that $CD8^+$ T cells, in which GATA3 is under-expressed, have adopted the Runx3-Eomes pathway for their optimal IFNγ production (Cruz-Guilloty et al., 2009).

Runx3 is highly expressed in Th1 cells and enforced expression of Runx3 DN in Th1 cells results in diminished IFNγ production consistent with an earlier report that Runx3 regulates IFNγ production (Djuretic et al., 2007). Here we show Runx3-mediated IFNγ production can be blocked by GATA3. Furthermore, by using ChIPseq, we show that, in normal Th1 cells, the Runx protein complex binds to multiple critical regulatory elements of *Ifng* gene, some of which are also bound to T-bet. STAT4 has also been reported to directly bind to the *Ifng* gene. Therefore, optimal IFNγ production and Th1 differentiation involves three pathways: IL-12-STAT4-, IFNγ-T-bet- and Runx3-mediated pathways. Each pathway makes some contribution to IFNγ production through direct action on the *Ifng* gene, and there is also crosstalk among these pathways. For example, STAT4 and T-bet have a synergistic effect on inducing IFNγ production; Runx3 and T-bet can interact with each other and bind to many sites in *Ifng* locus; T-bet may be responsible for up-regulating Runx3 expression; IL-12-STAT4 is partially responsible for T-bet up-regulation; T-bet down-regulates GATA3 during Th1 differentiation resulting in the up-regulation of STAT4 as well as the release of the inhibition of Runx3-mediated IFNγ production. Our finding of GATA3 regulating Runx3 function and STAT4, but not T-bet expression, increases our understanding on the cross-regulation of Th1 versus Th2 differentiation.

We also show that GATA3 physically interacts with Runx3 in primary T cells and the level of IFNγ production strongly correlates with the ratio of Runx3 to GATA3. The N-terminus and zinc fingers, but not C-terminus of GATA3, are required for the interaction with Runx3. The GATA3 mutant with the C-terminal deletion substantially loses its ability to induce IL-4, but is fully functional in suppressing Runx3-mediated IFNγ production, suggesting that the positive and negative functions of GATA3 can be separated. This may be critical for guiding effective immune intervention strategies in treating Th1 and Th2-related diseases.

Two reports previously suggest that the association of transcription factors dominant in different Th lineages can mutually regulate their functions (i.e. T-bet and GATA3; Foxp3 and RORγt) (Hwang et al., 2005; Zhou et al., 2008). Here, we reported a third example of such mutual regulation, between GATA3 and Runx3 during Th1-Th2 differentiation. Since both GATA3 and Runx3 are also critical during CD4 versus CD8 lineage commitment in thymus (Bosselut, 2004; Hernandez-Hoyos et al., 2003; Pai et al., 2003; Sato et al., 2005; Setoguchi et al., 2008; Taniuchi et al., 2002), the interaction and cross-regulation of these two molecules may also play a role in determining CD4-CD8 fate in the thymus, a subject that needs to be further studied.

In conclusion, Runx3 directly binds to many critical regulatory elements at *Ifng* locus and induces IFNγ expression even in the absence of T-bet. GATA3 suppresses Runx3-mediated IFNγ production through protein-protein interaction. Therefore, the relative amount of GATA3 and Runx3 expression regulates Th1 versus Th2 responses.

EXPERIMENTAL PROCEDURES

Animals

Mice carrying dLck-Cre transgene (Line 3779), *Runx3*$^{fl/fl}$ mice and *Gata3*$^{fl/fl}$ mice were previously reported (Naoe et al., 2007; Zhang et al., 2005; Zhu et al., 2004). *Tbx21*$^{-/-}$ mice (Line 4648) were obtained from Jackson laboratory (Finotto et al., 2002). All mice were bred

and maintained under specific pathogen-free conditions in the National Institute of Allergy and Infectious Diseases (NIAID) animal facility and used at 5–12 weeks of age under an approved protocol according to the NIAID guidelines for animal care.

Preparation of Cells

$CD4^+$ T cells were isolated from lymph nodes by negative selection as previously described (Yamane et al., 2005) except when autoMACS (Miltenyi Biotec) was used. For purification of naïve $CD4^+$ T cells, $CD44^{low}CD62L^{high}$ $CD4^+$ T cells were sorted by FACSAria (BD Biosciences). T cell-depleted splenocytes were prepared by incubation with anti-Thy1.2 mAb supernatant and rabbit complement (Cedarlane Laboratories Limited) at 37°C for 45 min followed by irradiation at 30 Gy (3,000 rad). In some experiments, mice were injected subcutaneously with 500 third-stage infectious larvae of *N. brasiliensis* (Zhu et al., 2004). On day 9 after *N. brasiliensis* infection, $CD4^+CD44^{high}CD62L^{low}$ T cells from mesenteric lymph nodes were sorted by FACSAria.

Activation of T Cells

$CD4^+$ T cells were cultured with irradiated T cell-depleted splenocytes in the presence of 1 μg/ml of anti-CD3 (145-2C11) and 3 μg/ml of anti-CD28 (37.51) for 3 days with various combinations of antibodies and cytokines: For Th1 conditions, 10 ng/ml of IL-12 and 10 μg/ml anti-IL-4 (11B11); for Th2 conditions, 5000 U/ml of IL-4, 10 μg/ml anti-IL-12 (C17.8) and anti-IFNγ (XMG1.2); for ThNeutral conditions, no additional cytokines or antibodies added. The activated cells were then cultured in IL-2 (50 U/ml) containing medium.

Retroviral (RV) Constructs and Infection

Eomes-GFP-RV and T-bet DN-GFP-RV constructs were previously described (Pearce et al., 2003). *Runx3* and *Gata3* cDNA were cloned from Th1 and Th2 cells, respectively, by PCR. The details for the construction of the Runx3-RV, GATA3-RV, Runx3 DN-RV and mutant GATA3-RV are described in Supplementary Experimental Procedures. Retroviruses were prepared by transfecting Phoenix-Eco packaging cell line with RV constructs using Fugene6 (Roche) as previously described (Zhu et al., 2004) and were concentrated from the culture supernatant by centrifugation at 12,000 g for 14–18 hour at 4°C. $CD4^+$ T cells were stimulated with T cell-depleted splenocytes for 24 hr in the presence of anti-CD3 and anti-CD28 mAbs under Th1 or Th2 conditions, and then infected with concentrated RV as previously described (Zhu et al., 2004). In some experiments, on day 4, RV-infected cells were sorted based on the expression of GFP and/or Thy1.1.

RNA Purification and Quantitative PCR

Total RNAs were isolated using a combination of TRIzol (Invitrogen) and RNeasy Kit (QIAGEN). cDNAs were prepared using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCR was performed on a 7900HT Sequence Detection System (Applied Biosystems) using the following pre-designed primer-probe sets: IL-4, IFNγ Eomes, T-bet, STAT4, IL-12Rβ2, CXCR3, GAPDH (all purchased from Applied Biosystems) and GATA3 as previously described (Zhu et al., 2004). Primers and probe for detecting distal Runx3 are: 5′-TCCAACAGCATCTTTGACTCCTT-3′, 5′-GGTGCTCGGGTCTCGTATGA-3′ and 5′-FAM-CCCAACTATACACCAACC-MGB-3′. The efficiency of *Gata3* deletion was determined by quantitative PCR as previously described (Zhu et al., 2004).

Intracellular Staining

Intracellular staining was performed as previously described (Zhu et al., 2004). Briefly, the activated cells maintained in IL-2-containing medium for 1–3 days were re-stimulated with10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 nM ionomycin in the presence of 2 mM

monensin for 4 hours. At the end of stimulation, cells were stained with anti-CD4 and anti-Thy1.1, washed and fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized in PBS containing 0.5% Triton X-100 and 0.1% BSA. They were then stained for cytokines and analyzed by FACSCalibur or LSR II (BD Biosciences) and results were analyzed using FlowJo software (Tree Star). Staining of the transcription factors were carried out with Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Anti-GATA3 (L50-823), anti-T-bet (4B10) and anti-Eomes (Dan11mag) were purchased from BD Biosciences and e-Biosciences, respectively.

ChIPseq Analysis

5×10^6 cells $CD4^+$ T cells that have been cultured under Th1 conditions for 2 rounds, with each round consisting of 4-day TCR stimulation and 2-day culture in IL-2-containing medium, were cross-linked with formaldehyde and the chromatin was sonicated into small fragments. Then the fragmented chromatin was immunoprecipitated with specific antibodies. Anti-CBFβ was kindly provided by Dr. I. Taniuchi. T-bet antibody (4B10) was purchased from Santa Cruz. The ChIP DNA fragments with size ~200bp were treated and sequenced using the Illumina-Solexa 1G Genome Analyzer as previously described (Barski et al., 2007). Sequence reads originating in 200 bp windows were summed and displayed as custom tracks on the UCSC Genome Browser.

Immunoprecipitation and Immunoblotting

$CD4^+$ T cells activated under Th1 or Th2 conditions for 4–5 days were used for immunoprecipitation and/or immunoblotting. In some experiments, RV constructs containing Runx3, WT and mutant GATA3 cDNAs were transfected into HEK 293T cells using Fugene6 and these cells were harvested 2 days after transfection. The details of immunoprecipitation and immunoblotting are described in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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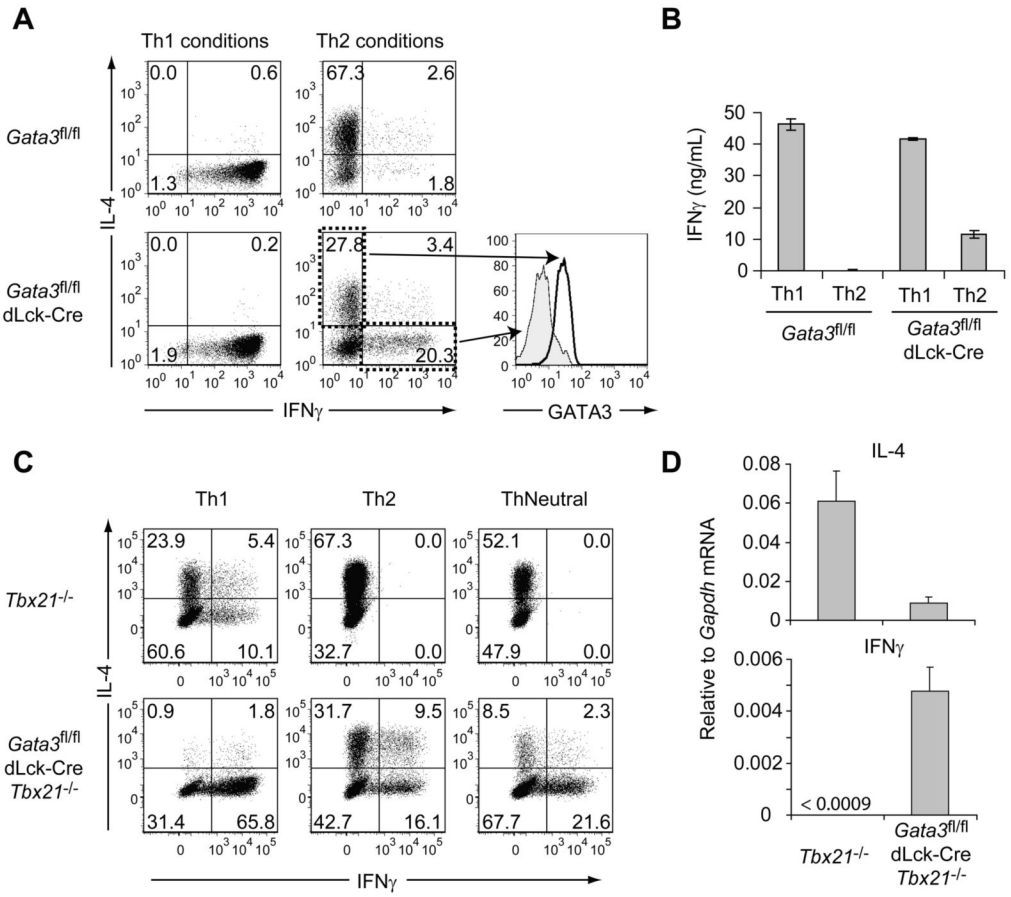

Figure 1. *Gata3* Deletion from Naïve CD4$^+$ Cells Results in T-bet-independent IFNγ Production during Th2 Differentiation both*in vitro* and *in vivo*

(A) CD4$^+$ T cells isolated from *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-dLck-Cre mice were activated under Th1 or Th2 conditions in the presence of T-depleted splenocytes for 3 days. After cultured with IL-2-containing medium for another 2 days, the cells were re-stimulated with PMA plus ionomycin for 4 hrs. The expression of IL-4, IFNγ and GATA3 were measured by intracellular staining. Numbers indicate the percentage in each quadrant. (B) The amount of IFNγ protein in the supernatants of the cells prepared in (A) that had been re-stimulated with PMA and ionomycin for 24 hrs were measured by ELISA. Error bars represent means ± SD. (C) Naive CD4$^+$ T cells isolated from *Tbx21*$^{-/-}$ or *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ mice were activated under Th1, Th2 or ThNeutral conditions and analyzed as in (A). (D) Nine days after *N. brasiliensis* infection, CD44^{high}CD62L^{low} CD4$^+$ T cells were sorted from mesenteric lymph nodes of the infected mice and quantitative RT-PCR was performed. The relative expression of IL-4 and IFNγ was normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Error bars represent means ± SD of the data from three individual mice in each group. Data are representative of three independent experiments (A and C).

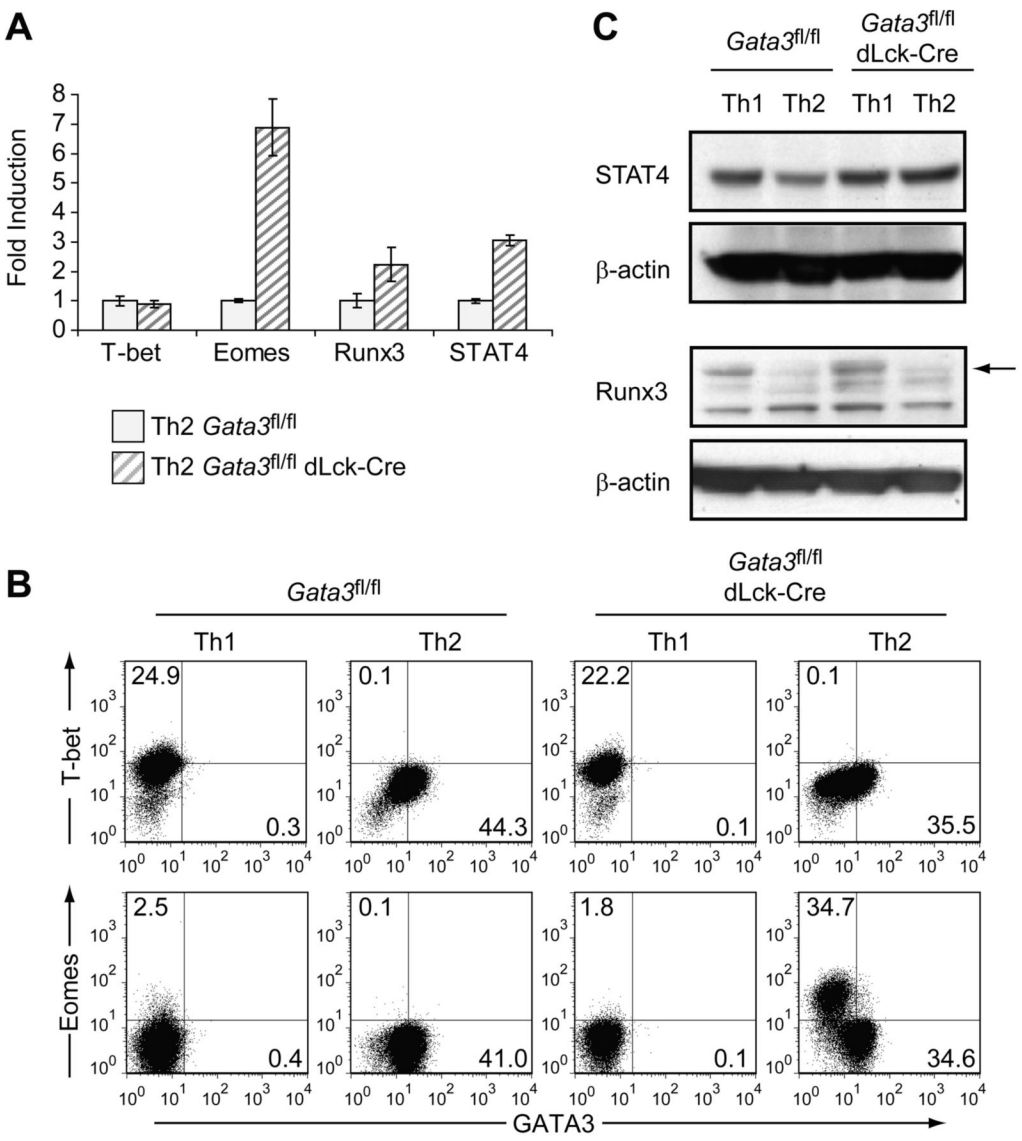

Figure 2. Increase of Eomes, Runx3 and STAT4 but not T-bet Expression in *Gata3*$^{fl/fl}$-dLck-Cre CD4^{+} T Cells Differentiated under Th2 Conditions

(A) Total RNAs from *Gata3*$^{fl/fl}$ or *Gata3*$^{fl/fl}$-dLck-Cre CD4^{+} T cells cultured under Th2 conditions were prepared. The expression of indicated genes was measured by quantitative RT-PCR. The relative expression was normalized by the expression of GAPDH and the expression of each gene in WT Th2 cells was set as "1". Error bars represent means ± SD. (B) Th1 and Th2 cells prepared as in Figure 1A were stained for intracellular Eomes, T-bet, and GATA3 expression. Numbers indicate the percentage in each quadrant. (C) STAT4 and Runx3 protein expression in the cells prepared in (B) was measured by western blotting. Arrow indicates Runx3 protein. Data are representative of three independent experiments.

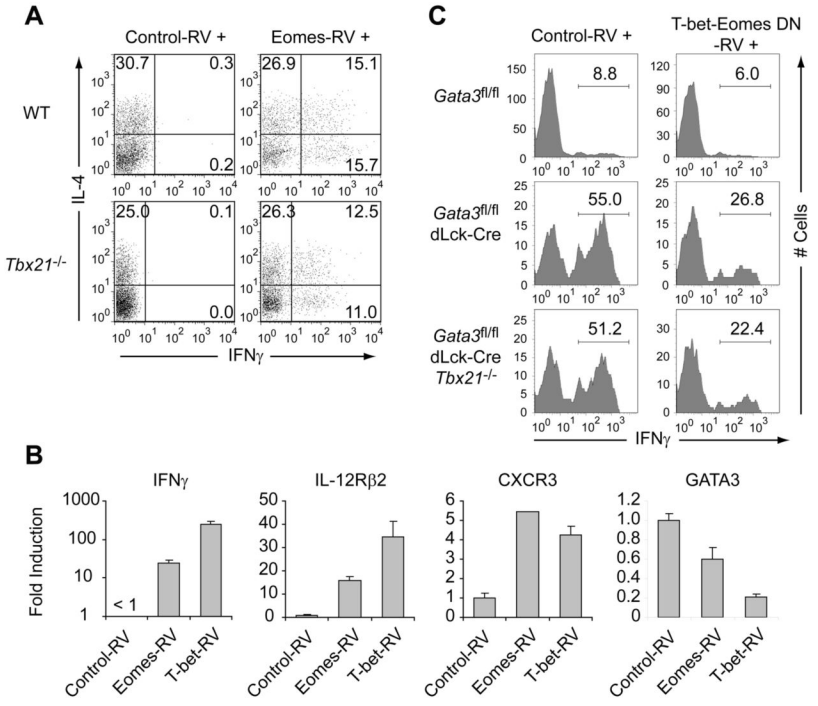

Figure 3. Eomes but not T-bet Contributes to the IFNγ Production in *Gata3*$^{fl/fl}$-dLck-Cre "Th2" Cells

(A) CD4$^+$ T cells isolated from WT or *Tbx21*$^{-/-}$ mice were stimulated under Th2 conditions and infected with Eomes-GFP-RV (Eomes-RV) or empty GFP-RV as a control (Control-RV). (B) Naive CD4$^+$ T cells isolated WT mice were stimulated under Th2 conditions and infected with Control, Eomes or T-bet-GFP-RV. GFP$^+$CD4$^+$ T cells from each group were sorted and quantitative RT-PCR was performed. The relative expression was normalized by GAPDH and the expression of each gene in Control-RV infected Th2 cells was set as "1". Error bars represent means ± SD. (C) CD4$^+$ T cells isolated from WT, *Gata3*$^{fl/fl}$-dLck-Cre or *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ mice were stimulated under Th2 conditions and infected with T-bet-Eomes DN-GFP-RV (a RV containing a dominant negative form of T-bet) or Control-GFP-RV. (A and C) Activated cells were re-stimulated with PMA plus ionomycin and intracellular staining of IFNγ and IL-4 were performed. The plots were gated on CD4$^+$GFP$^+$ cells. Numbers indicate the percent of cells in each quadrant or gate. Data are representative of two independent experiments.

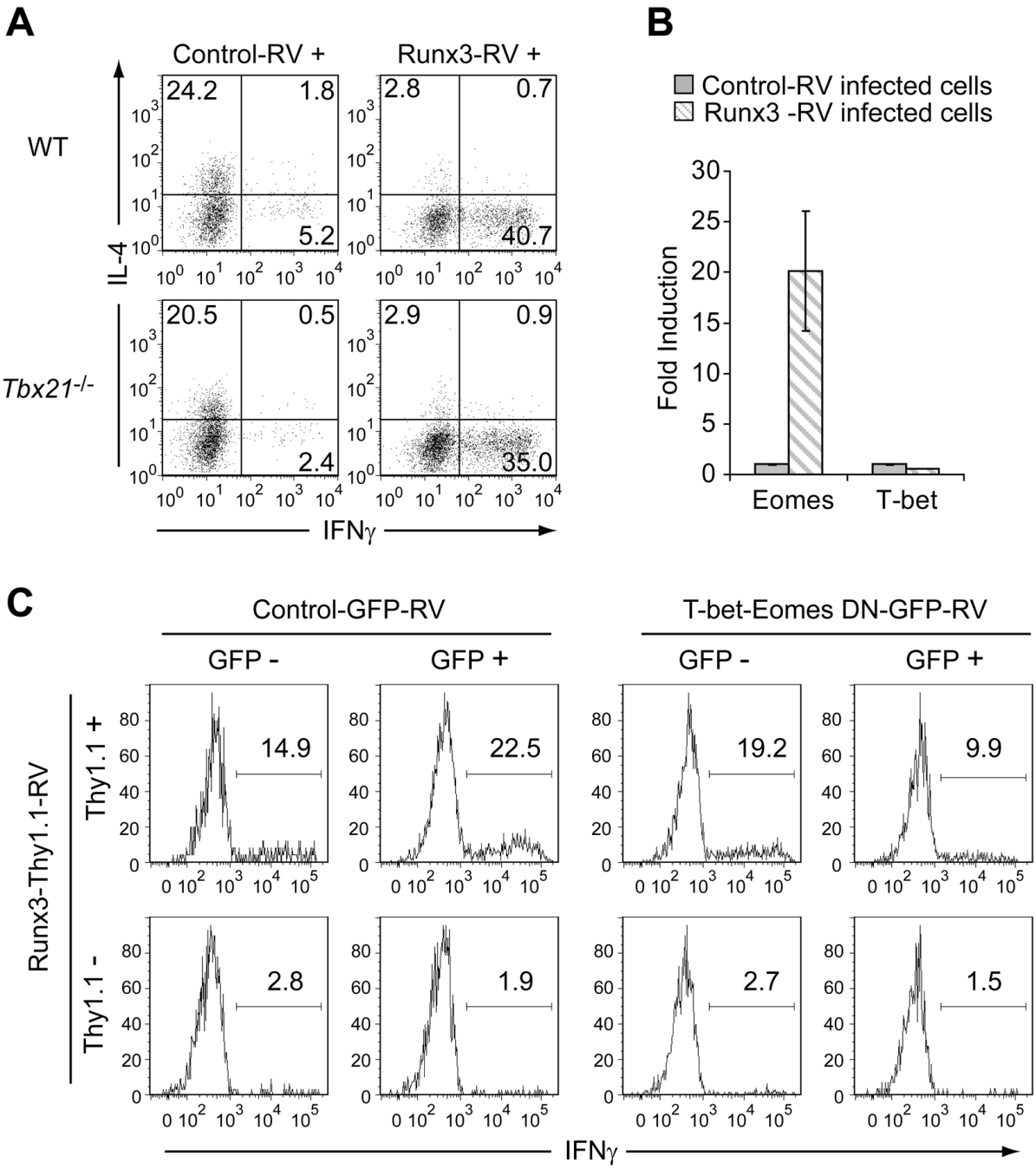

Figure 4. Runx3 Induces IFNγ Production and Eomes expression

(A) $CD4^+$ T cells isolated from WT and *Tbx21*$^{-/-}$ mice were stimulated under Th2 conditions and infected with Runx3-GFP-RV or Control-GFP-RV. Infected cells were re-stimulated and stained as in Figure 3A. The dot plots were gated on $CD4^+GFP^+$ cells. (B) $CD4^+GFP^+$ cells were isolated by cell sorting from the cells prepared in (A). RNAs were prepared from sorted cells and the expression of Eomes and T-bet was measured by quantitative RT-PCR. The expression levels of each gene in Control-RV infected cells were set as "1". (C) $CD4^+$ T cells isolated from WT mice were stimulated under Th2 conditions and then co-infected with Runx3-Thy1.1-RV and T-bet-Eomes DN-GFP-RV or Control-GFP-RV. Infected cells were re-stimulated and stained as in Figure 3A. Numbers indicate the percent of cells in each quadrant

or gate. Error bars represent means ± SD. Data are representative of two (B) or three (A and C) independent experiments.

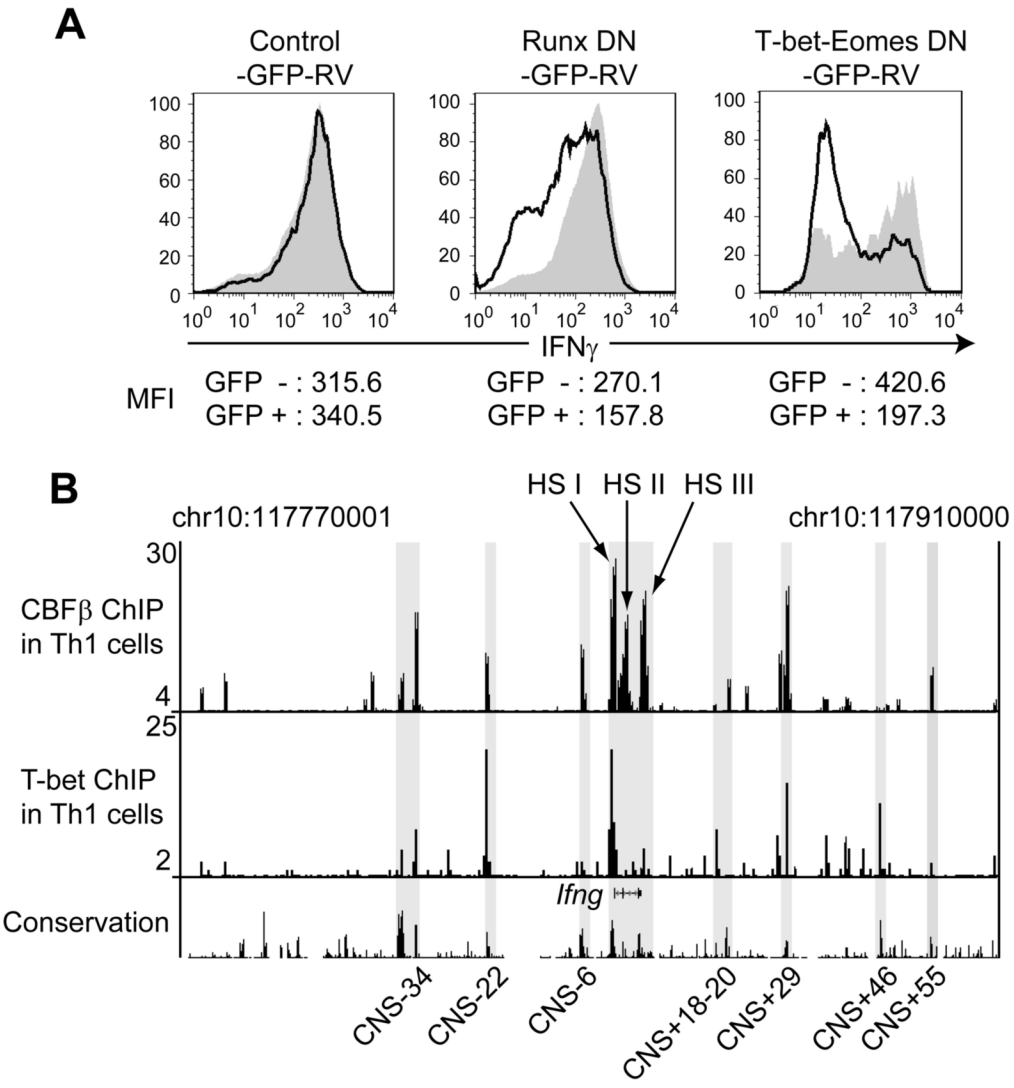

Figure 5. Runx3 is Important for Optimal IFNγ Production by Th1 cells and It Binds to Many Regulatory Elements Located in *Ifng* Locus

(A) WT $CD4^+$ T cells were stimulated under Th1 conditions and infected with Runx3 DN-GFP-RV, T-bet-Eomes DN-GFP-RV or Control-GFP-RV. Infected cells were re-stimulated and stained as in Figure 3A. The black lines represent $CD4^+GFP^+$ cells and the shaded area represents $CD4^+GFP^-$ cells in the same culture. (B) WT Th1 cells were harvested and fixed. ChIPSeq analysis was performed using specific antibody against CBFβ or T-bet. The sequence tags mapped to the locus of *Ifng* are shown. Critical Runx and T-bet binding elements corresponding to DNase I hypersensitivity (HS) sites and conserved non-coding sequences (CNS) are highlighted.

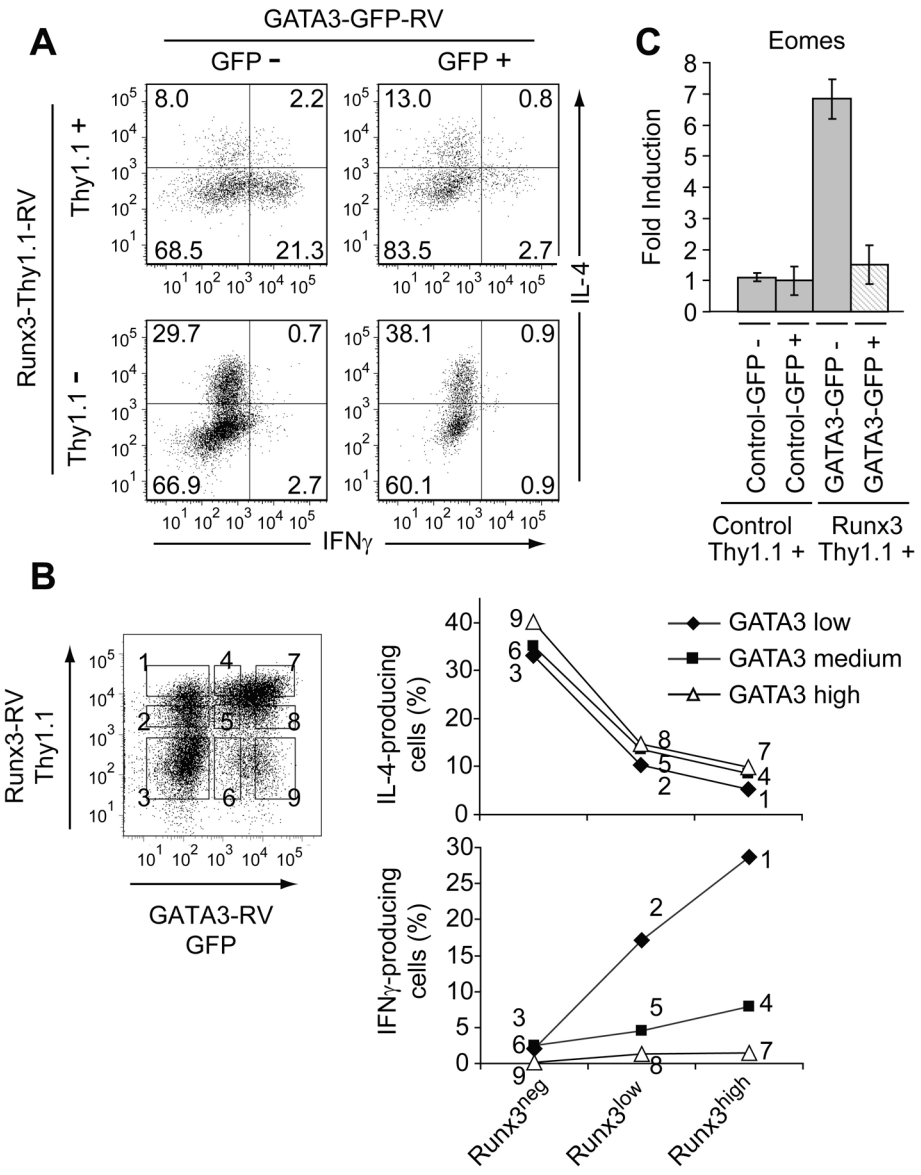

Figure 6. GATA3 Represses Runx3-Mediated IFNγ Production and Eomes Induction

(A and B) WT CD4$^+$ T cells were stimulated under Th2 conditions and co-infected with GATA3-GFP-RV and Runx3-Thy1.1-RV. Infected cells were re-stimulated and stained as in Figure 3A. The dot plots were gated based on the expression of GFP and Thy1.1 (A). Nine subsets (1 through 9) expressing different amount of GFP and Thy1.1 were selected for further analysis (B, left panel). The percentages of IL-4 or IFNγ-producing cells from these 9 subsets were plotted (B, right panel). (C) WT CD4$^+$ T cells were stimulated and infected as in (A). Infected cells were sorted based on the expression of GFP and Thy1.1. RNAs were prepared from sorted cells and the expression of Eomes was measured by quantitative RT-PCR. The relative expression of Eomes in Control-Thy1.1$^+$ and Control-GFP$^+$ cells was set as "1". Error bars represent means ± SD. Data are representative of three (A and B) and two (C) independent experiments.

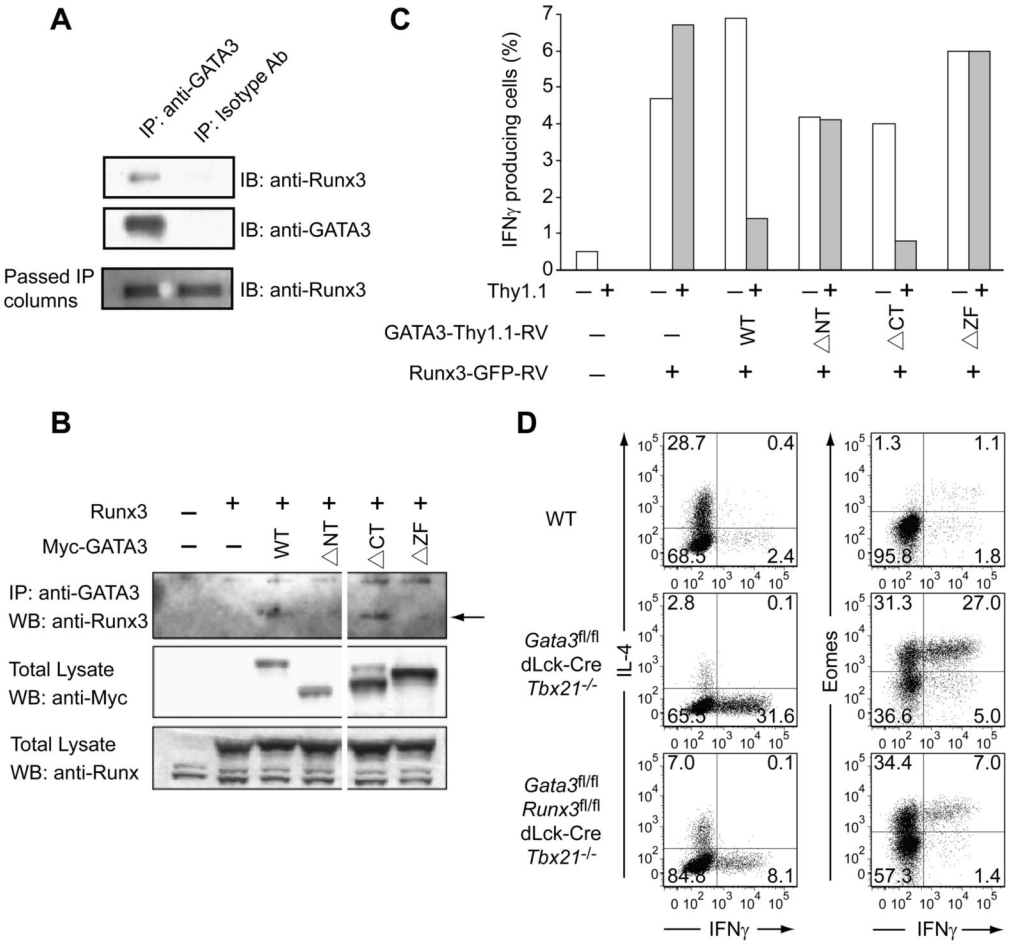

Figure 7. Runx3 Protein Interacts with GATA3 Protein and Runx3 is Responsible for IFNγ Production in GATA3-deficient "Th2" Cells

(A) Total Th1 cell lysates were immunoprecipitated with anti-GATA3 or isotype antibody and then immune-blotted with anti-Runx polyclonal Ab. The membrane was then stripped and re-blotted with GATA3 mAb. The cell lysates passed the IP columns were also blotted with anti-Runx as a loading control. (B) Runx3-RV and Myc-tagged WT or mutant GATA3-RV were co-transfected into HEK 293T cells as indicated. Total cell lysates or anti-GATA3-immunoprecipitated cell lysates were blotted with anti-Runx polyclonal Ab or anti-Myc. (C) WT $CD4^+$ T cells were stimulated under Th2 conditions and co-infected with Runx3-GFP-RV and WT or mutant GATA3-Thy1.1-RV. Infected cells were re-stimulated and stained as in Figure 3A. The percentage of IFNγ-producing cells in each group was plotted. (D) $CD4^+$ T cells isolated from WT, *Gata3*$^{fl/fl}$-dLck-Cre-*Tbx21*$^{-/-}$ or *Gata3*$^{fl/fl}$*Runx3*$^{fl/fl}$ dLck-Cre-*Tbx21*$^{-/-}$ mice were activated under Th2 conditions as shown in Figure 1A. They were then stained for intracellular IL-4, IFNγ and Eomes expression. Numbers indicate the percentage in each quadrant. Data are representative of two independent experiments.