Reciprocal regulation of the Il9 locus by counteracting activities of transcription factors IRF1 and IRF4

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The T helper 9 (Th9) cell transcriptional network is formed by an equilibrium of signals induced by cytokines and antigen presentation. Here we show that, within this network, two interferon regulatory factors (IRF), IRF1 and IRF4, display opposing effects on Th9 differentiation. IRF4 dose-dependently promotes, whereas IRF1 inhibits, IL-9 production. Likewise, IRF1 inhibits IL-9 production by human Th9 cells. IRF1 counteracts IRF4-driven Il9 promoter activity, and IRF1 and IRF4 have opposing function on activating histone modifications, thus modulating RNA polymerase II recruitment. IRF1 occupancy correlates with decreased IRF4 abundance, suggesting an IRF1-IRF4-binding competition at the Il9 locus.

Furthermore, IRF1 shapes Th9 cells with an interferon/Th1 gene signature. Consistently, IRF1 restricts the IL-9-dependent pathogenicity of Th9 cells in a mouse model of allergic asthma. Thus our study reveals that the molecular ratio between IRF4 and IRF1 balances Th9 fate, thus providing new possibilities for manipulation of Th9 differentiation.

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The generation of T helper (Th) subsets enables specific targeting of pathogens. Signals triggered by antigen recognition, costimulation and cytokines lead to the activation and differentiation of naïve T cells by inducing a network of interacting transcription factors that guide their differentiation into distinct Th subsets. The expression of hallmark cytokines characterizes each subset and outlines their specific effector properties1. Interferon (IFN)-γ-producing Th1 cells express the master regulator T-bet and promote clearance of intracellular pathogens, whereas Th2 cells secreting interleukin (IL)-4, IL-5 and IL-13 are characterized by the master transcription factor GATA3 and contribute to immunity against helminths. IL-17-, IL-21- and IL-22-producing Th17 cells depend on the lineage-specific transcription factor retinoic acid–related orphan receptor-γt (RORγt) and have a fundamental function in protection from extracellular bacterial and fungal infections. However, Th cell subsets can exert both beneficial and detrimental effects; Th1 and Th17 cells have been implicated in autoimmune tissue inflammation, and Th2 cells can contribute to allergy and asthma1–3. Furthermore, although Th1 cells (characterized by IL-9 production) are involved in immunity against helminth6 and antitumour responses2–8, these cells also contribute to immunopathologies, including asthma10–12, atopic dermatitis13, autoimmune14 and colitis15. Hence, unraveling the transcriptional network that regulates Th9 differentiation is pivotal for understanding protective as well as pathogenic effects in atopic and autoimmune diseases.

Th9 cell differentiation is dictated by the cytokine transforming growth factor-β (TGF-β) in combination with IL-4 (refs 6,16), cytokines that shape the transcriptional Th9 network in concert with T-cell receptor (TCR)-induced and IL-2-induced signals. TGF-β-induced PU.1 binds directly to the Il9 promoter and probably enhances IL-9 production by modulating permissive histone acetylation at the Il9 locus10,17. CD4 + T cells deficient in IL-2 do not produce IL-9 and this defect can be reversed by the addition of exogenous IL-2, which induces signal transducer and activator of transcription factor 5 (STAT5)-mediated activation of the Il9 promoter18–20. IL-4 via STAT6 signalling positively regulates Th9 differentiation by enhancing Il9 promoter activity21,22 and by upregulating the transcription factor GATA3, which promotes Th9 fate16,23. Furthermore STAT6 signalling counteracts the IL-9-suppressing transcription factor Foxp3 (refs 16,24,25). Importantly, IL-2/STAT5 (ref. 26) and IL-4/STAT6 (ref. 22) as well as TCR signalling27 promote the expression of interferon regulatory factor 4 (IRF4), which is essential for Th9 differentiation11.

The IRF family of transcription factors consists of nine members; each IRF comprises of a well-conserved DNA-binding domain (DBD), but most IRFs also contain an IRF association domain, which is responsible for homologous as well as heterologous interactions27. Compared to other members of the IRF family, IRF4 has lower affinity for the consensus binding motif termed interferon-stimulated response elements (ISRE). IRF4 rather binds cooperatively with other transcription factors to composite regulatory elements28,29. In conjunction with the activator protein 1 (AP-1) family member BATF, IRF4 binds preferentially to AP-1/IRF4 composite element (AICE) motifs30–32, whereas complexes of IRF4 and proteins from the ETS family, including PU.1, interact at ETS-IRF composite element (EICE) motifs34,35. IRF4 and BATF are crucial factors for Th9 differentiation12 and consequently, IRF4- or BATF-deficient mice are resistant to Th9-dependent allergic airway disease11,12. The importance of IRF4 is further demonstrated in T cells deficient in the tyrosine kinase Itk, which is an important component of TCR-mediated signalling. Altered TCR signalling in these cells leads to IL-9 inhibition due to attenuated IRF4 expression, which can be rescued by IL-2/STAT5-mediated IRF4 induction26. Hence, IRF4 has not only a fundamental role in the differentiation of Th9 but is also known to control Th2, Th17, T follicular helper and T regulatory cell specification27.

The first member of the IRF family, IRF1, favours Th1 differentiation in an intrinsic manner by enhancing the expression of IL-12 receptor β1 subunit16 and also in a T-cell-extrinsic manner by increasing IL-12 production by antigen-presenting cells37–38. Conversely, IRF1 suppresses the production of IL-4, IL-5 and IL-13 in Th2 cells39,40. Thus IRF1 balances Th1 versus Th2 differentiation, and dysregulated IRF1 consistently associates with atopy41 and allergic asthma42, immunopathologies linked to both Th2 and Th9 cells. IRF1 is expressed at low levels by most cell types and is induced by various stimuli, including IL-1, lipopolysaccharide, tumour necrosis factor and IFNs. In Th2 cells, IFN-γ stimulation in combination with TCR signalling, strongly upregulates IRF1 expression leading to its increased binding to ISRE, thus inhibiting IL-9 production19. Likewise, IFN-γ is known to inhibit IL-9 production via STAT1 (refs 19,43); however, the exact cellular mediators downstream of STAT1 are not known44.

Three conserved non-coding sequences (CNS) have been described for the Il9 locus, CNS0, 1 and 2, whereby the promoter (CNS1) harbours important transcription factor-binding sites for Il9 regulation45. Among them, several ISRE motifs can be found and IFN1 as well as IRF4 bind to the Il9 promoter9,11. Considering these findings and that both IRF1 and IRF4 can be upregulated in Th9 cells9,11, we speculated on a yet unknown interference between these two IRFs during Th9 differentiation.

Here we provide evidence that IFN-γ-mediated upregulation of IRF1 limits IL-9 production in human and mouse Th9 cells. IRF1 suppresses IRF4-driven IL-9 production and counteracts IRF4-driven Il9 promoter transactivation. Decreased IRF4 abundance correlates with IRF1 binding at the Il9 locus, suggesting possible competitive binding among these factors. Furthermore, IRF4 and IRF1 display opposing activities on the chromatin state, thus possibly contributing to differential RNA polymerase II recruitment to Il9. Genome-wide analyses reveal that IRF1 imprints Th9 cells with a Th1/IFN-associated gene signature, while suppressing Th9 genes. Consistently, IRF1 suppresses Th9 cell-mediated allergic airway disease in an IL-9-dependent manner. Collectively, our data reveal direct opposing activities between IRF members on Th9 differentiation.

**Results**

**IRF1 suppresses IRF4-dependent IL-9 production in Th9 cells.** To examine whether there is a mutual regulation between IRF1 and IRF4 expression in Th9 cells, we determined IRF1 and IRF4 protein levels in the presence or absence of IFN-γ, which is a known IRF1 inducer46. As expected, IRF1 was strongly upregulated by IFN-γ in Th9 cells (Fig. 1a,b) in a STAT1-dependent manner (Supplementary Fig. 1a,b). The upregulation of IRF1 by IFN-γ did not affect the levels of co-expressed IRF4 and vice versa (Fig. 1a,b). Furthermore, neither the expression of several transcription factors involved in Th9 differentiation10,12,16,18,21–25 including PU.1 (encoded by Sp1), BATF, GATA-3 and T-bet nor the phosphorylation levels of STAT3 and STAT5 were significantly affected by IRF1-deficiency (Supplementary Fig. 1c–g). The expression of Foxp3, which represses Th9 differentiation16,22,25 was slightly upregulated in IRF1−/− as compared to wild-type (WT) Th9 cells (Supplementary Fig. 1h).

Although IRF1 did not interfere with the levels of IRF4 and other transcription factors contributing to Th9 differentiation,
it was still conceivable that it affects IL-9 expression in Th9 cells by other mechanisms. To test this possibility, we sorted naive CD44−CD62L+CD4+ T cells from WT, Irf4−/−, Irf1−/− or Stat1−/− mice and then treated under Th9 (TGF-β and IL-4) or Th0 (without skewing cytokines) conditions with/without IFN-γ as indicated. (a, b, c) After resting and reculture under Th9 conditions for 2 days, intracellular flow cytometric analysis for IRF1 and IRF4 were performed. Bars give geometric mean of fluorescence intensity (MFI). (c) Cells were restimulated and then IL-9 and IFN-γ production was detected by flow cytometry, bars to the right give mean of IL-9+ and IFN-γ+ cells. (d) Irf4−/− or Stat1−/− CD4+ T cells were isolated, activated under Th0 condition overnight and then spin-infected with retroviruses as indicated: IRF4-GFP-RV, control-Thy1.1-RV, and IRF1-Thy1.1-RV. Thereafter, cells were cultured under Th9 conditions for 2 days, rested for 3 days and recultured under Th9 conditions for additional 2 days. Four subsets (I–IV) were selected for analysis of IL-9 production. (e) Bars show fold induction of IL-9+ T cells relative to GFP+ Thy1.1+ cells (subset I). (f) Six subsets (a through f) expressing increasing levels of GFP and Thy1.1 were selected for analysis of IL-9 production (left panel). Dot plot to the left is representative for four independent experiments. Graph to the right shows fold induction of IL-9+ T cells relative to GFP+ Thy1.1+ cells (subset a) combined from four independent experiments. Histogram and contour-plots are representative of two (a, b), three (c) or four (d, e) independent experiments. Bars show mean ± s.d. from combined two (a, b), three (c) or four (d, e) experiments. *P<0.05, **P<0.005, ***P<0.001 (two-tailed Student’s t-test).

Figure 1 | IRF1 limits IRF4-driven IL-9 production dose-dependently. (a–c) Naive CD44−CD62L+CD4+ T cells were isolated from WT, Irf4−/−, Irf1−/− or Stat1−/− mice and then treated under Th9 (TGF-β and IL-4) or Th0 (without skewing cytokines) conditions with/without IFN-γ as indicated. (a, b) After resting and reculture under Th9 conditions for 2 days, intracellular flow cytometric analysis for IRF1 and IRF4 were performed. Bars give mean of IL-9 induction of IL-9+ cells (fold) IL-9+ cells (fold). (c) Cells were restimulated and then IL-9 and IFN-γ production was detected by flow cytometry, bars to the right give mean of IL-9+ and IFN-γ+ cells. (d) Irf4−/− or Stat1−/− CD4+ T cells were isolated, activated under Th0 condition overnight and then spin-infected with retroviruses as indicated: IRF4-GFP-RV, control-Thy1.1-RV, and IRF1-Thy1.1-RV. Thereafter, cells were cultured under Th9 conditions for 2 days, rested for 3 days and recultured under Th9 conditions for additional 2 days. Four subsets (I–IV) were selected for analysis of IL-9 production. Bars show fold induction of IL-9+ T cells relative to GFP+ Thy1.1+ cells (subset I). (e) Six subsets (a through f) expressing increasing levels of GFP and Thy1.1 were selected for analysis of IL-9 production (left panel). Dot plot to the left is representative for four independent experiments. Graph to the right shows fold induction of IL-9+ T cells relative to GFP+ Thy1.1+ cells (subset a) combined from four independent experiments. Histogram and contour-plots are representative of two (a, b), three (c) or four (d, e) independent experiments. Bars show mean ± s.d. from combined two (a, b), three (c) or four (d, e) experiments. *P<0.05, **P<0.005, ***P<0.001 (two-tailed Student’s t-test).
IRF1 and IRF4 control the Il9 locus. Since IRF4 and most transcription factors related to Il9 locus regulation exert their function through direct binding to the Il9 promoter, we analysed IRF4 and IRF1 binding and their functional interference at the Il9 promoter. For this, we performed reporter assays in non-polarized CD4+ T cells transfected with an Il9 promoter-luciferase construct (pGL3-Il9) and IRF1- and/or IRF4-encoding plasmids. Co-transfection of the Il9 promoter-luciferase construct with IRF4 resulted in transactivation (Fig. 2a), as previously described. Consistent with the repressive activity of IRF1 on IRF4-mediated IL-9 production, the co-transfection of IRF1 suppressed IRF4-driven Il9 promoter activity in non-polarized CD4+ T cells (Fig. 2a). The inhibitory IRF1 effect was dose-dependent and relied on the DBD (Fig. 2b,c), suggesting that IRF1 might directly interfere with the transactivating effects of IRF4 at the Il9 promoter. We further confirmed the role of IRF1 DBD on IL-9 production in Th9 cells using overexpression of retroviral vectors encoding IRF1DBD (IRF1DBD-GFP-RV), IRF1 (IRF1-GFP-RV) or control vector (Control-GFP-RV) (Supplementary Fig. 3a,b).

In order to evaluate the underlying molecular mechanisms of IRF1- and IRF4-dependent Il9 regulation, we performed chromatin immunoprecipitation (ChIP) with IRF4- and IRF1-specific antibodies in Th9 cells followed by sequencing (ChIP-Seq) as described. To determine the permissive histone marks of regions bound by IRF1 or IRF4, we included ChIP-Seq analyses for acetylated histone H3 at Lys27 (H3K27-Ac), which marks of regions bound by IRF1 or IRF4, we included ChIP-Seq analysis (Fig. 2g). We found that IRF1 peaks highly correlated with ISRE occurrence in Th9 cells (Fig. 3c, Supplementary Fig. 4a). This was accompanied by a decreased pol II recruitment to the Il9 gene upon IFN-γ treatment in WT Th9 cells (Fig. 3d) and this effect was reversed by IRF1 deficiency (Fig. 3d). This implies that negative regulation of histone acetylation state by IRF1 may contribute to the restricted Il9 transcription. In contrast, IFN-γ treatment suppressed H4-Ac at Il9 CNS in WT but not in IRF1+/- Th9 cells (Fig. 3c, Supplementary Fig. 4a). This was accompanied by a decreased pol II recruitment to the Il9 gene upon IFN-γ treatment in WT Th9 cells, and this effect was reversed by IRF1 deficiency (Fig. 3d).

IRF1 imprints Th9 cells with IFN-γ/-Th1-associated signature. On a genome-wide level, IFN-γ signalling caused both hypoacetylation and hyperacetylation on loci in Th9 cells, indicating a differential regulation of regulatory regions (Fig. 4a). To understand the extent to which IRF1 contributes to transcriptional programming of Th9 cells on a global level, we performed RNA sequencing (RNA-Seq) from WT or Irf1+/- Th9 cells treated with IFN-γ. There were 735 significantly downregulated, while 604 significantly upregulated transcripts (P<0.01) in Irf1+/- relative to WT Th9 cells (Fig. 4b), indicating that IRF1 signalling negatively and positively regulates genes downstream of IFN-γ. Gene-set enrichment analysis (GSEA) using Molecular Signatures Database (MSigDB, Broad Institute) as well as published gene expression signatures.
revealed that positively regulated genes by IRF1 signalling (those upregulated in WT as compared to Ifr1Δ/− Th9 cells) were enriched for IFN-γ response genes and Th1 signature-associated genes (Fig. 4c, Supplementary Fig. 5a,b). In contrast, the negatively regulated genes (those downregulated in WT cells as compared to Ifr1Δ/− Th9 cells) were enriched for genes associated with a Th9 signature (Fig. 4e, Supplementary Fig. 5c). Hence, IFN-γ–IRF1 signalling promotes a transcriptional shift of Th9 cells towards an IFN-γ/Th1 signature. This was further confirmed by functional enrichment analysis on sets of genes derived from IRF1 promoter-biding profiles. A majority of pathways linked to the IFN/Th1 signature overlapped between GSEA and ChiP-Seq-based gene ontology (GO) enrichment analysis of IRF1 targets, including viral response pathways as well as IL-12- and IFN-associated pathways (Supplementary Data 1). Among the genes positively regulated by IRF1 signalling and directly bound by IRF1, we found the already known targets, guanylate-binding protein 2 (Gbp2) and Gbp5 (refs 50,51), which contribute to clearance of infection as well as Th1-associated genes, including Il12rb1 (refs 1,36; Fig. 4f). Collectively, the data suggest that IRF1 can act as a direct activator of IFN/Th1 pathways, implying that IRF1 signalling modulates the transcriptional fate of Th9 cells by enriching IFN-γ/Th1-associated gene signatures, while limiting Th9-associated genes.

**IRF1 limits allergic airway inflammation IL-9 dependently.**

As IRF1 single-nucleotide polymorphisms associate with childhood allergic asthma⁴² and IL-9 production is postulated to be an initial event promoting allergy⁵², we analysed the role of IRF1 in a murine model of allergic asthma. However, allergy studies directly in Ifr1Δ/− mice will not be informative due to defects in antigen-presenting cells,⁴³ in addition to T-cell-intrinsic alterations. To circumvent this difficulty, we polarized WT and Ifr1Δ/− OTII cells, carrying a transgenic TCR specific for chicken ovalbumin (OVA), under Th9 conditions in the presence of IFN-γ. Thereafter, the cells were injected into mice lacking T and B cells (Rag2Δ/−Δ), which were subsequently challenged with OVA to provoke asthma symptoms (Supplementary Fig. 6a). Ifr1Δ/− compared to WT OTII Th9 cells caused significantly increased eosinophil numbers in broncho-alveolar lavage (BAL) fluid, while macrophage numbers were decreased (Fig. 5a).
The experiments were repeated (as determined by using control IgG) were subtracted. Chromatin was used for control ChIP experiments with control IgG. Precipitated DNA is presented relative to input (% input). Values for nonspecific binding of H4-Ac relative to WT Th9 cells treated with IFN-γ/C0 Irf1 were scored fragments per kilobase of exon per million fragments mapped (FPKM). massive parallel sequencing of genes. (upper lanes) or anti-H3K27Ac antibody (middle and bottom lanes). Transcription start site of genes are indicated below as arrows.

**Figure 3 | Opposite effects of IRF1 and IRF4 on histone acetylation at Il9 locus.** (a-d) Purified WT and (a,b) Irf4−/− or (c,d) Irf1−/− CD4+ T cells were cultured under Th9 conditions with or without IFN-γ. (a,c) ChIP analysis of histone H4 acetylation (Ac) at CNS of the Il9 gene. (c) Bars show fold induction of H4-Ac relative to WT Th9 cells treated with IFN-γ. (b,d) ChIP analysis for RNA polymerase II (pol II) occupancy at Il9 and Rpl32 genes. (a-d) The same chromatin was used for control ChIP experiments with control IgG. Precipitated DNA is presented relative to input (% input). Values for nonspecific binding (as determined by using control IgG) were subtracted. (a-d) Data are shown as mean ± s.d. of combined results from two independent experiments (triplicates to quintuplets in qRT-PCR). The experiments were repeated (b) three or (d) five times with consistent results. *P<0.05, **P<0.005, ***P<0.001 (two-tailed Student’s t-test).

**Figure 4 | The IFN-γ/IRF1 pathway upregulates IFN/Th1-associated genes in Th9 cells.** (a) ChIP-Seq analyses of Th9 cells with or without IFN-γ treatment. Cells were crosslinked with formaldehyde and immunoprecipitated with anti-H3K27-Ac antibodies. Massive parallel sequencing of immunoprecipitated DNA was performed. Numbers in the Venn diagram represent H3K27-Ac peaks. (b) Naive CD4+ T cells isolated from WT and Irf1−/− mice were cultured under Th9 conditions in the presence of IFN-γ. Total RNA was purified from the cells and RNA-Seq was performed (three independent biological samples). Heatmap shows colour-coded z-scored fragments per kilobase of exon per million fragments mapped (P<0.01). (b-d) GSEA was performed according to the Broad institute with (c) the hallmark IFN-γ response, (d) Th1 upregulated genes and (e) Th9 upregulated genes. (f) ChIP-Seq analysis of Th9 cells with or without IFN-γ treatment. Cells were crosslinked with formaldehyde and immunoprecipitated with anti-IRF1 (upper lanes) or anti-H3K27Ac antibody (middle and bottom lanes). Transcription start site of genes are indicated below as arrows.
This was accompanied by increased presence of mucus-producing cells in recipients of Irf1−/− compared to WT OTII Th9 cells (Fig. 5b). Additionally, OVA-stimulated lung isolated from recipients of Irf1−/− compared to WT OTII Th9 cells produced more IL-9 and IL-13 but less IFN-γ (Fig. 5c–e), revealing that Irf1−/− Th9 cells retain their pro-allergic phenotype in vivo as compared to WT Th9 cells. The pro-allergic function of Irf1−/− Th9 cells was ameliorated by treatment with IL-9-neutralizing antibodies as determined by decrease in eosinophilia, in the numbers of mucus-producing cells and in inflammation (Fig. 5f–h, Supplementary Fig. 6b,c). Accordingly, the amounts of secreted IL-9 and IL-13 (which is regulated by IL-9 in asthma43) were strongly diminished by IL-9 neutralization (Supplementary Fig. 6d,e). Thus IFN-γ/Irf1 signalling restricts Th9-mediated airway allergy in an IL-9-dependent manner while enhancing IFN-γ production in Th9 cells, indicating that Irf1 constrains allergic airway disease by skewing Th9 cells towards a 'low-allergic' IFN/Th9 phenotype.

IRF1 suppresses IL-9 production in human Th9 cells. Finally, we investigated whether Irf1 also regulates IL-9 production in human Th9 cells. For this, we isolated naïve CD4+CD25−CD45RA+CD45RO− T cells from peripheral blood mononuclear cells (PBMCs) of healthy human subjects and activated them in the absence of skewing cytokines (Th9 conditions) or in the presence of TGF-β plus IL-4 (Th9 conditions) with or without addition of IFN-γ. As already reported13,44, Th9 conditions induced IL-9 production by CD4+ T cells, whereas the addition of IFN-γ counteracted this effect and simultaneously upregulated IFN-γ (Fig. 6a). This was confirmed by qRT-PCR and detection of IL-9 in supernatants of cultured cells (Fig. 6b,c). Similar to murine Th9 cells (Supplementary Fig. 1a), the addition of IFN-γ caused increased phosphorylation of STAT1 also in human Th9 cells (Fig. 6d) and this was accompanied by upregulation of Irf1, which was co-expressed with IRF4 (Fig. 6e). Knockdown of IRF1 expression by siRNA (Fig. 2f) abolished IFN-γ-mediated suppression of IL9 mRNA expression and IL-9 production in human Th9 cells (Fig. 6g,h). Thus IFN-γ signalling requires IRF1 to suppress IL-9 production in human Th9 cells. This points to the existence of comparable programmes dictated by the IFN-γ/STAT1-IRF1 pathway suppressing IL-9 production in human and murine Th9 cells.

Discussion
Th9 cells exert protective as well as pathophysiological immune functions44; however, the detailed regulation of the Th9 transcriptional network remains incompletely understood. Here we show that IRF1 limited IL-9 production downstream of IFN-γ/STAT1 signalling in mouse and human Th9 cells. IRF1 repressed IRF4-driven IL-9 production and IL9 promoter activity in a quantitative manner. Direct IRF1 binding at the IL9 promoter was associated with decreased abundance of IRF4 and reduced acetylation levels as well as limited RNA polymerase recruitment at the IL9 locus. These results suggest that the suppressive function of IRF1 is probably mediated by a direct binding competition with IRF4 at the IL9 locus, resulting in opposite regulation of histone acetylation and associated IL9 transcription. IFN-γ-induced IRF1 evoked a shift in Th9 cells towards an IFN/Th1-like signature through direct and indirect mechanisms. Accordingly, the absence of Irf1 in IFN-γ-treated Th9 cells led to increased IL9-dependent pathogenicity in AAD, a mouse model for allergic asthma. Thus the transcriptional network of Th9 differentiation is shaped by opposing actions of two IRF family members, IRF1 and IRF4.

Th9 cells contribute to human atopic diseases and accordingly Th9-related genes associate with asthma development44.
We demonstrated that IFN-γ upregulates IRF1 in human Th9 cells and that IRF1 upregulation correlated with decreased IL-9 production. Furthermore, silencing of IRF1 restored IFN-γ-mediated IL-9 suppression, indicating that IRF1 counteracts IL-9 production also in human Th9 cells. Indeed, IRF1 single-nucleotide polymorphisms associate with asthma development and atopy. In addition, increased levels of IRF4 and IL-9 were accompanied by diminished IFNG expression in the peripheral blood of allergic asthmatics. Given those findings, one could speculate that asthmatics may fail to upregulate IRF1 upon infection-induced IFN-γ signalling leading to exacerbated asthma due to defects in Th9 and Th2 inhibition. However, further work must be carried out to fully understand the impact of IRF1 signalling on Th9 cells during asthma exacerbations.

To induce asthma pathophysiology in the mouse model, we performed adoptive transfer experiments of OVA-transgenic IFN-γ-treated WT and Irf1−/− Th9 cells into Rag2−/− mice and subsequently challenged the mice with nebulized OVA. Indeed, the absence of IRF1 resulted in more severe AAD, which was dependent on IL-9 as shown by neutralization with anti-IL-9 antibody. Besides decreasing IL-9 production, we wondered whether IRF1 as a transcription factor linked to Th1 differentiation also affects other functionally relevant genes. Therefore, we made use of genome-wide RNA-Seq to investigate the complex IRF1-mediated transcriptional interplay in IFN-γ-treated Th9 cells. Interestingly, GSEA revealed an IRF1-dependent and IFN-γ-induced shift towards a Th1/IFN-like phenotype, while suppressing Th9 signature genes. Pathways associated with infectious and interferon response, for example, Gbp and l2rβ1 were directly upregulated, indicating that IRF1 balances Th9 versus Th1-like plasticity by its ability to act as a transcriptional repressor as well as an activator. Recently, Végran et al. published that IRF1 is a positive regulator of IL-9 and Th9 differentiation when induced by IL-1β and dictates IL-21-dependent antitumour function in a melanoma model. However, there are many possible explanations for this discrepancy to our study. The cytokine signalling triggered by IL-1β differs greatly from that of IFN-γ, therefore it is conceivable that the cellular milieu contains distinct transcription factors that might interfere with IRF1 functions. Among these, nuclear localization of the IL-9-promoting transcription factor nuclear factor-kB is induced by IL-1β signalling, which might alter IRF1 actions. To exclude the influence of the microbial environment of the animal facilities, we extended our work to human Th9 cells where we demonstrated that IRF1 indeed inhibited IL-9 expression in the context of IFN-γ signalling. We therefore conclude that IFN-γ-IRF1 signalling suppresses the Th9 fate in humans and mice.

IRF family members are known to regulate gene expression positively or negatively in different cell types, including Th cells. To carry out these effects, IRFs can interact via the IRF association domain with family members or with proteins belonging to other families. For example, IRF1–IRF8 heterodimers mostly act as transcriptional repressors at ISRE, whereas IRF1, IRF3 and IRF7 form an IFN-β enhanceosome together with nuclear factor-kB, AP-1 and CBP/p300 to induce Ifnb expression. Only few studies describe antagonizing functions of IRFs at ISRE in gene regulatory. It has been described that IRF8 represses IRF9 binding to ISRE at IFN-stimulated gene 15 (ref. 29), while IRF4 counteracted IRF1-driven tumour necrosis factor-related apoptosis-inducing ligand promoter activity in a DBD-dependent manner. Furthermore, IRF1 opposes IRF4-driven Il17 promoter activity, and conversely, IRF4 antagonizes IRF1-mediated Il10 promoter activity.
In our study, we propose a model in which IRF1 competes with IRF4 binding at ISRE of the Il9 locus to repress IL-9 expression. This is substantiated by (i) dose-dependent IRF1-mediated repression of IRF4-driven Il9 promoter activity, (ii) dose-dependent suppression of IRF4-triggered IL-9 by overexpressed IRF1 in Th9 cells and (iii) decreased IRF4 binding in the presence of IRF1 occupancy at the Il9 locus as shown by ChIP-Seq analyses. The previously defined IRF4-binding region in the Il9 promoter harbors several ISRE (AANNGAAA) motifs, which we analysed for IRF4-promoting function. We identified two functional IRF4-binding sites at −238/-226bp from the transcription start site on the basis of Il9 promoter activity assays performed with mutated ISRE. This was surprising because IRF4 peaks associated preferentially with AICE motifs on a genomewide scale. As IRF4 (and IRF8), in comparison to other IRF family members including IRF1, displays lower affinity for binding to ISRE, we presume those sites to be competitively bound by IRF1 leading to opposing regulation of Il9 promoter activity in Th9 cells. Transcription factor-induced transactivation is a multilevel event. After histone methylation-based initiation of active chromatin formation by pioneering factors, ensuing transcription factors such as EP300 enable the recruitment of transcriptional coactivator complexes, which then catalyse local histone acetylation (H3K27Ac) required for gene transcription. Both IRF1 and IRF4 have been shown to affect the histone acetylation status in immune cells. We indeed detected opposite effects of IRF4 and IRF1 on the histone H4 acetylation status at Il9 regulatory elements. Thus histone acetylation was decreased in IRF4-deficient Th9 cells as compared to WT Th9 cells, suggesting that IRF4 contributes to increased chromatin accessibility at the Il9 locus. In contrast, IRF1 was crucial for IFN-γ-mediated suppression of permissive histone marks, implying that IRF1 limited the chromatin accessibility at the Il9 CNS. Consistent with these counteracting activities, the recruitment of RNA pol II, which is crucial for transcriptional output, was decreased in IRF4-deficient Th9 cells, while it was increased under IRF1-deficiency as compared to WT cells. This points out that the changes in the acetylation status may contribute to the opposing regulation of IL-9 production by IRF4 and IRF1.

The model in which two members of one family competitively bind at the same cytokine locus has already been demonstrated for STAT3 and STAT5 in Th17 cells. In this study, IL-2 signalling mediated its repressing effects via direct DNA binding of STAT5 at the Il17 locus, which correlated with decreased STAT3 abundance as well as decreased H3-Ac. This effect was mediated by increased binding of a histone deacetylase adaptor protein termed NcoR2. In our study, the general HDAC inhibitor trichostatin A was insufficient to oppose IFN-γ-mediated IL-9 repression, suggesting that the IFN-γ-IRF1 axis suppresses IL-9 production in an HDAC-independent manner. As IRF4 is known to recruit HAT p300 in Th0 and Th17 cells, this could also apply for Th9 cells. Along with this idea, one could speculate that IRF1 counteracts the IRF4-mediated HAT recruitment or interferes with HAT function. However, more work has to be done to clarify this issue in detail. A recent study pointed to competitive binding of IRF1 and IRF4 at the Il17 promoter thereby possibly regulating Th17/Th1 commitment. This is in agreement to our proposed model of IRF1/IRF4 competition as a mechanism to control Th differentiation.

Takakeno et al., our study offers deeper insight into the function of IRF1 and IRF4 in Th9 cell differentiation and suggests for the first time that IRF1 competes with IRF4 to repress IL-9 production in Th9 cells. Furthermore, we demonstrate that IRF1 regulated gene expression positively on some, whereas negatively on other loci to shift Th9 cells towards a Th1-like phenotype. The knowledge of this IRF1-mediated transition of Th9 differentiation could prove useful for further unravelling Th9- and IFN-γ-associated immune responses during asthma as well as anti-tumour immunity. Altering the IRF1/IRF4 balance in the transcriptional network of Th9 cells to either increase or decrease Th9 responses might display a potential therapeutic approach.

Methods

Mice. C57Bl/6 mice were purchased from The Jackson Laboratory. Ifr1−/−, Rag2−/−, and OTII mice expressing a transgenic TCR recognizing OVA232-339 were bred at the animal facility of the Biomedical Research Center, University of Marburg. Ifr1−/− mice were bred with OTII mice to produce Ifr1−/−×OTII transgenic mice. Stat1−/− mice were kindly provided by T. Decker (Max F. Perutz Laboratories, Department of Genetics, Microbiology and Immunology, University of Vienna, Austria). All mice used in the experiments were 8–12-week old, at C57Bl/6 background and sex- and age-matched. The experiments were approved by the local committee (Regierungspräsidium Gießen) and conducted according to the German animal protection law.

Murine CD4+ T cell purification and in vitro differentiation. CD4+ T cells were purified by negative magnetic cell sorting (CD4+ T cell isolation kit, 130-104-454, Miltenyi) from the spleens and lymph nodes of 8–12-week-old mice. For some experiments, cells were negatively purified CD4+ T cells were further sorted on a FACS AriaIII (BD Biosciences) to obtain naive CD4−CD62L+CD44−CD25−. CD4+ T cells using anti-CD4-V450 (RM4-5, BD Biosciences), anti-CD44-PE (IM7, eBioscience) and anti-CD62L-Alexa Fluor 700 (MEL-14, BD Pharmingen) monoclonal antibodies (mAbs) in a dilution of 1:500. Sorting purity was typically >97% in post-analysis. The cells were primed with plate-bound anti-CD3 mAb (3 mg·ml−1, 145-2C11) and anti-CD28 mAb (5 mg·ml−1, 37.51, both mAbs produced and purified ‘in house’) in the presence of recombinant human (rh) IL-2 (50 U·ml−1, Novartis) and anti-mouse-IFN-γ (5 ng·ml−1, XMG1.2, produced and purified ‘in house’). The cells were cultured under neutral (Th0) conditions, by the addition of anti-IL-4 (10% culture supernatant of clone 11B11), or Th9 conditions, by the addition of rhIFN-β (1.5 ng·ml−1) and rmIL-4 (20 ng·ml−1). Some cultures were supplemented with recombinant rat IFN-γ (5 ng·ml−1, Peprotech) as indicated in the figure legends. Cells were usually harvested on day 2 for intracellular staining and qRT-PCR analysis. For kinetic experiments, the cells were analysed as indicated in figure legends.

For adoptive transfer experiments, CD4+ T cells were purified by negative magnetic cell sorting (CD4+ T cell isolation kit, 130-104-454, Miltenyi) from the spleens and lymph nodes of 8–12-week-old OTII and Ifr1−/−×OTII transgenic mice. The cells were differentiated under Th9 conditions in the presence of anti-mIL-4 (5 mg·ml−1) and rat IFN-γ (5 mg·ml−1). After 2 days, the cells were harvested, washed, counted and 5 × 106 WT or Ifr1−/−×OTII Th9 cells were transferred by intraperitoneal (i.p.) injection into Rag2−/− mice.

Intracellular staining. For intracellular cytokine staining after differentiation, cells were restimulated with phosphor myristate acetate (50 mg·ml−1) and ionomycin (1 μg·ml−1) in the presence of brefeldin A (5 μg·ml−1, both from Sigma) for 4 h, fixed, stained with anti-CD4 (RM9A4, Biolegend) and anti-IFN-γ-phycoerythrin (PE) (XMG 1.2, eBioscience) mAbs. For intracellular staining of transcription factors after differentiation, cells were fixed with a Foxp3/Transcription Factor Fixation/Permeabilization Concentrate and Diluent (eBioscience) and then stained with: anti-Foxp3 PE (FJK-16, eBiosciencie), anti-T-bet-PE (eBioscience), anti-GATA3-eF660 (TWAJ, eBioscience), anti-IRF4-AIF647, (J64, eBioscience) or anti-IRF1-PE (D564, Cell Signaling) mAbs. All antibodies were used in a 1:500 dilution. The cells were analysed by flow cytometry using a FACS Calibur or a FACSariaIII (BD Biosciences) with the DIVA software (BD, Biosciences) or the FlowJo Software (Tree Star).

Phospho-flow. For staining of phosphorylated STAT1, human Th9 cells were harvested after 48 h of culture, rested for 4 h and treated with rhIFN-γ (100 ng·ml−1) for 20 min. Then the cells were fixed and permeabilized according to BD Phosflow Protocol III using Lyse/Fix Buffer (557870, BD) and Perm Buffer III (558050, BD) and stained with anti-human phospho-STAT1 (Y701)-eFluor660 (KIKS0083, eBioscience, 1:100).

Stimulation and differentiation of human naive CD4+ T cells. PBMCs from healthy donors were labelled with anti-CD4-Pacific Blue (RPA-T4, BioLegend), anti-CD45RA-AMCA (HT100, eBiosciences), anti-CD45RO-PE (UCHL1, BD) and anti-CD25-allophycocyanin (BC06, eBioscience) in 1:500 dilutions, and then naive CD4+ T cells (CD4+ CD45RA+ CD45RO− CD25−) were sorted using a FACS AriaIII (BD), the purity was typically >97% in post-sort analysis. Naive CD4+ T cells were cultured in RPMI complete media (3% human AB serum) with rhIL2 (30 U·ml−1) and activated with Dynabeads Human T-Activator CD3/CD28 (111.31D, Invitrogen, T cell/bead = 2:1). For induction of Th9 cell differentiation,
rhIL-4 (30 ng ml⁻¹) and rhTGFB-β1 (5 ng ml⁻¹) and either anti-HIF-1γ (5 µg ml⁻¹) or eIF2α-Ser70 (50 ng ml⁻¹) were added to the cell cultures. Th0 cells were differentiated in the presence of anti-hIL-4 and anti-HIF-1γ (both 5 µg ml⁻¹). Cells were analysed by flow cytometry for IRF1 and IRF4 presence after 24–36 h of culture and for IL-9 and IFN-γ production after 72 h of culture; supernatants were collected after 72 h and IL-9 was measured by enzyme-linked immunosorbent assay (ELISA; human IL-9 ELISA Max, 434705, BioLegend).

RNA-mediated interference. An Amaxa Human T cell Nucleofector Kit (VPA 1062, Lonza) was used for knockdown of human IRF1 by siRNA in naive CD4⁺ T cells according to the manufacturer’s instructions. Naive CD4⁺ T cells (5 × 10⁵) were activated with Dynabeads Human T-Activator CD3/CD28 overnight and then transduced with 3 µM IRF1-specific siRNA (ONTARGETplus, L011704-01) or control scrambled RNA (both Dynarmon) by the T-23 transfection program (Amaxa). Thereafter, the cells were cultured under Th9 conditions for additional 24–48 h, followed by flow cytometric analysis and evaluation of IL-9 mRNA by qRT-PCR and IL-9 production by ELISA.

Immunoblot analysis. Purified murine CD4⁺ T cells were left unstimulated or stimulated as indicated in the figure legends. For detection of Tyr959/STAT5 and Tyr610STAT6 phosphorylation, cells were preactivated under Th9 conditions in the presence of anti-mIFN-γ (5 µg ml⁻¹) and/or anti-HIF-1γ (5 ng ml⁻¹) for 2 days, then washed and rested in cytokine-free medium for 8 h. Preactivated cells were treated with rabbit anti-P-STAT6 (5 ng ml⁻¹) in combination with either mRl-2 (20 ng ml⁻¹) or rh-2 (50 µl ml⁻¹) for 20 or 60 min. Whole-cell lysates were prepared as described previously⁴⁷, 20 µg of total protein were loaded per lane and proteins were detected according to standard protocols. The following Abs were used: anti-β-actin (AC-15, Sigma-Aldrich), anti-IRF1 (M-20, Santa Cruz), anti-IRF-4 (M-17, Santa Cruz), anti-STAT1 (99172, Cell Signaling), anti-STAT5 (C-17, Santa Cruz), anti-P-STAT6 (Y641, SC-11762, Santa Cruz), anti-goat IgG-HRP (#sc-2055, Santa Cruz), anti-mIFN-γ (5 ng ml⁻¹), anti-mIFN-β (5 ng ml⁻¹), anti-mIL-13 (50 ng ml⁻¹) and anti-rabbit IgG-HRP (a-859, Cell Signaling). qRT-PCR with the primers: forward: 5'-AGG AGT TAA AGA TCT AGC CCC AAC CCC CTT-3' and reverse: 5'-CTC ACC AGG AAC CAG CAG-3'. The PCR product was control digested with BglII and EcoRI and cloned into the retroviral vector pMSCV containing the IRES-regulated gene for GFP or Thy-1. The construct was sequenced to prove authenticity. The retroviral vector pMSCV containing IRF4-IRES-GFP, the empty control vector containing IRES-GFP and transduction were described previously⁴⁷, 5T, IRF4 or IRF1 CD4⁺ T cells were activated with Dynabeads Human T-Activator CD3/CD28 overnight and then rested under Th9 conditions and, on day 1, were infected with an IRF1-expressing retrovirus (IRF1-RV), IRF1 mutant-expressing retrovirus (IRF1ADBD-RV), IRF4-expressing retrovirus (IRF4-RV) or a control retrovirus (Control-RV). Transfected cells were cultured under Th9 conditions (TGF-β and IL-4) for 48 h, then washed and rested in media containing rhIL-2 (50 U ml⁻¹) and mIFN-γ (5 U ml⁻¹) for 72 h. Thereafter, the cells were re-cultured under Th9 conditions for additional 48 h, then washed, restimulated and analysed by flow cytometry for intracellular IL-9, or without restimulation, the cells were analysed for intracellular IRF1 and IRF4 levels.

Chromatin immunoprecipitation. Th9 cells cultured for 2 days in the presence of anti-mIFN-γ (5 µg ml⁻¹) and/or rat IFN-γ (5 ng ml⁻¹) as indicated in the figure legends. In all, 2–5 × 10⁷ cells were crosslinked with 1% formaldehyde for 6 min at room temperature. ChIP was performed as described previously⁴⁷. Lysed cells were sonicated in a Bioruptor Plus (Diagenode) with 30 s ON, 30 s OFF on high power output for 27–33 cycles at 4°C. For immunoprecipitation, 2.5–4 µg of the following Abs were used: anti-IRF1 (M-20, sc-640x, Santa Cruz), anti-STAT1 (9172, Cell Signaling), anti-RNA pol II (N-20, sc-899x, Santa Cruz), anti-H4-AcD1/8 (06-866, Millipore) and control IgG (#2729, Cell Signaling). qRT-PCR with the primers: forward: 5'-GAG AGT TAA AGA TCT AGC CCC AAC CCC CTT-3' and reverse: 5'-CTC ACC AGG AAC CAG CAG-3'. The PCR product was control digested with BglII and EcoRI and cloned into the retroviral vector pMSCV containing the IRES-regulated gene for GFP or Thy-1. The construct was sequenced to prove authenticity. The retroviral vector pMSCV containing IRF4-IRES-GFP, the empty control vector containing IRES-GFP and transduction were described previously⁴⁷, 5T, IRF4 or IRF1 CD4⁺ T cells were activated with Dynabeads Human T-Activator CD3/CD28 overnight and then rested under Th9 conditions and, on day 1, were infected with an IRF1-expressing retrovirus (IRF1-RV), IRF1 mutant-expressing retrovirus (IRF1ADBD-RV), IRF4-expressing retrovirus (IRF4-RV) or a control retrovirus (Control-RV). Transfected cells were cultured under Th9 conditions (TGF-β and IL-4) for 48 h, then washed and rested in media containing rhIL-2 (50 U ml⁻¹) and mIFN-γ (5 U ml⁻¹) for 72 h. Thereafter, the cells were re-cultured under Th9 conditions for additional 48 h, then washed, restimulated and analysed by flow cytometry for intracellular IL-9, or without restimulation, the cells were analysed for intracellular IRF1 and IRF4 levels.

Asthma adaptive transfer model. Rag2-/-/- mice were injected i.p. with 5 × 10⁶ OT-II-Th9 cells on day 0 by i.p. injection. Mice were then challenged via the airways with nebulized OVA (1% in saline) with an ultrasonic nebulizer (NE-U17, Omron, Hooephoud, The Netherlands) for 20 min daily from day 1 to day 6. To neutralize IL-9 cytokine in vivo, mice were injected i.p. either with 30 µg anti-IL-9 (MP9, Millipore, produced and purified ‘in house’) or rat IgG (Sigma) antibody on days 1, +1 and +3 +5. On day 7, BAL and lung tissue were collected.

BAL and lung single-cell preparation. Cells were isolated by lavage of the lungs via a tracheal tube with PBS (1 ml). Numbers of BAL cells were counted with trypan blue dye exclusion. Differential cell counts were made from cytocentrifuged samples. For RNA Seq and bioinformatic analysis, naïve CD4⁺ T cells isolated from WT and Irf1⁻/⁻ mice were cultured for 2 days under Th9 conditions in the presence of IFN-γ (5 ng ml⁻¹). RNA was purified with the RNeasy Plus Minic Kit according to the manufacturer’s protocol (Qiagen). RNA was quantified with a Qubit 2.0 fluorometer (Invitrogen) and the quality was assessed on a Bioanalyzer 2100 (Agilent Technologies). Samples with an RNA integrity number of >8 were used for library preparation. Barcoded mRNA-seq cDNA libraries were prepared from 400 ng of total RNA using NEBNext Poly(A) mRNA
Magnetic Isolation Module and NEBNext Ultra RNA Library Prep Kit for Illumina according to the manual. Quantity was assessed using the Qubit HS Assay Kit and purity was determined using the Bioanalyzer 2100 Bioanalyzer. RNA sequencing data was performed with the Illumina HiSeq2500 using the HiSeq Rapid SBS Kit v2 (50 Cycle). The raw output data of the HiSeq was preprocessed according to the Illumina standard protocol. Quality control on the sequencing data was performed with the FastQC tool (available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), as well as the comprehensive Qorts suite. Inspecting the produced reports, all samples were deemed of good quality for further processing. Short reads alignment was performed with the ENSEMBL Mus.musculus.GRCm38 chosen as the reference genome. The corresponding annotation (ENSEMBL v76) was also retrieved from the ENSEMBL FTP site (http://www.ensembl.org/info/data/ftp/index.html). The STR aligner (version 2.4.0) was used to perform mapping to the reference genome. Alignments were processed with the featureCounts function of the Rsubread package, using the corresponding annotation (ENSEMBL v76) was also retrieved from the ENSEMBL FTP site. Quality control on the sequencing data was performed with the FastQC tool (available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), as well as the comprehensive Qorts suite. Inspecting the produced reports, all samples were deemed of good quality for further processing. Short reads alignment was performed with the ENSEMBL Mus.musculus.GRCm38 chosen as the reference genome. The corresponding annotation (ENSEMBL v76) was also retrieved from the ENSEMBL FTP site (http://www.ensembl.org/info/data/ftp/index.html). The STR aligner (version 2.4.0) was used to perform mapping to the reference genome. Alignments were processed with the featureCounts function of the Rsubread package, using the corresponding annotation (ENSEMBL v76) was also retrieved from the ENSEMBL FTP site. What is the null hypothesis that was tested in this study? The null hypothesis that was tested in this study is that the transcription factor IRF1 dictates the IL-21-dependent antitumor functions of TH9 cells. What is the statistical significance of this finding? The statistical significance of this finding is that the transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. What is the biological significance of this finding? The biological significance of this finding is that TH9 cells can express the transcription factor PU.1, which drives the development of IL-9-producing T cells and allergic inflammation.
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Authors contribution
L.C.C. and M.K. designed and performed in vitro and in vivo experiments;
Y.K. performed ChiP-Seq analyses; F.M. performed bioinformatical analyses; C.L., C.K.
and A.K. performed in vitro experiments; S.H.-B. performed in vivo experiments; S.S.,
M.L., F.B.-S., B.S., H.G., D.R. and A.B. provided suggestions for the study; T.B. and M.H.
oversaw, performed and designed the study and experiments, analysed data and,
together with L.C.C., wrote the manuscript.

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