Immunity
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# Interleukin-4 Production by Follicular Helper T Cells Requires the Conserved *Il4* Enhancer Hypersensitivity Site V

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DOI 10.1016/j.immuni.2011.12.014

## SUMMARY

**Follicular helper T cells (Tfh cells) are the major producers of interleukin-4 (IL-4) in secondary lymphoid organs where humoral immune responses develop. *Il4* regulation in Tfh cells appears distinct from the classical T helper 2 (Th2) cell pathway, but the underlying molecular mechanisms remain largely unknown. We found that hypersensitivity site V (HS V; also known as CNS2), a 3′ enhancer in the *Il4* locus, is essential for IL-4 production by Tfh cells. Mice lacking HS V display marked defects in type 2 humoral immune responses, as evidenced by abrogated IgE and sharply reduced IgG1 production in vivo. In contrast, effector Th2 cells that are involved in tissue responses were far less dependent on HS V. HS V facilitated removal of repressive chromatin marks during Th2 and Tfh cell differentiation and increased accessibility of the *Il4* promoter. Thus, Tfh and Th2 cells utilize distinct but overlapping molecular mechanisms to regulate *Il4*, a finding with important implications for understanding the molecular basis of allergic diseases.**

## INTRODUCTION

Type 2 immune responses entail a humoral response characterized by interleukin-4 (IL-4)-dependent IgE and IgG1 production, and cellular responses in peripheral tissues that are coordinated by T helper 2 (Th2) cells and innate immune cells that produce the signature Th2 cell-type cytokines IL-4, IL-5, and IL-13 (Voehringer et al., 2004). Type 2 immune responses have an important role in protective immunity against parasitic infections (Else et al., 1994), but when inappropriately exaggerated and misdirected to harmless antigens, cause allergic diseases such as asthma (Kay, 2001a, 2001b; Kim et al., 2010). Finding biological modifiers of the Th2 cell-type cytokines has emerged as a rational approach in developing new treatments for asthma (Levine and Wenzel, 2010). A complete mechanistic understanding of the molecular details of Th2 cell-type cytokine gene regulation may facilitate the development of novel approaches for therapeutic gene silencing in allergic diseases.

Elegant studies with cytokine gene reporter mice identified T cell subsets and innate immune cells that produce Th2 cell-type cytokines during type 2 immune responses in vivo (King and Mohrs, 2009; Neill et al., 2010; Price et al., 2010; Reese et al., 2007; Reinhardt et al., 2009; Saenz et al., 2010; Voehringer et al., 2004, 2006; Zaretsky et al., 2009). Follicular helper T (Tfh) cells have emerged as the major class of IL-4-producing T cells in the lymph node, and the IL-4 produced by these cells is critically required for shaping type 2 humoral immunity (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). The *trans*-acting factors required for IL-4 production by Tfh cells are distinctly different (GATA3- and STAT6-independent) from conventional Th2 cells, and the *cis*-regulatory requirements remain unknown (Reinhardt et al., 2009).

Gene expression in eukaryotes is tightly regulated by the chromatin structure of the underlying gene locus, which in turn influences the accessibility of *trans*-acting factors and the core transcriptional machinery to their binding sites in proximal gene promoters as well as distal *cis*-regulatory DNA elements (Berger, 2007; Li et al., 2007). Under physiological conditions, cell type specificity of gene expression is primarily conferred by distal *cis*-regulatory elements (Heintzman et al., 2009; Visel et al., 2009a, 2009b). A number of such elements were identified in the extended (~200 kb) murine Th2 cell-type cytokine locus spanning the *Il4*, *Il5*, and *Il13* genes and the constitutively

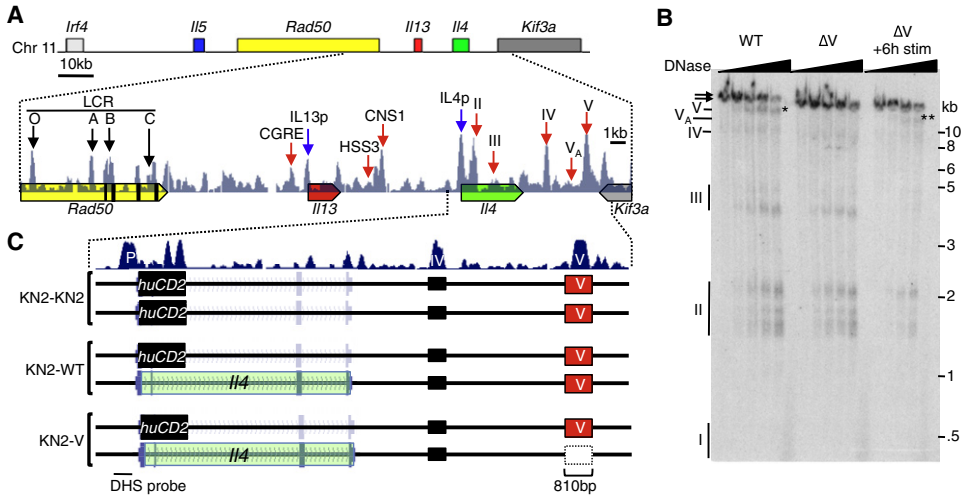


**Figure 1. *cis*-Regulatory Regions in the Mouse Th2 Cell Locus and Chromatin Analysis of HS V-Deficient T Cells**

(A) Diagram represents the murine Th2 cell-type cytokine locus showing locations of the DNase I hypersensitivity sites (HS) and conserved noncoding sequences (red arrows), locus control region (LCR, black arrows), *Il13* and *Il4* promoter (blue arrows), and species conservation tracks.

(B) DNase I HS analysis of unstimulated wild-type (WT) and ΔV (ΔV) Th2 cells either left unstimulated or stimulated for 6 hr with PMA and ionomycin (+6h stim) to induce HS $V_A$. Southern blot with a 5′ IL-4 probe revealed the indicated HS sites. Double arrow shows parent BamHI fragment. Note the HS V deletion decreases the size of this band, but not HS fragments in ΔV T cells. HS V and HS $V_A$ fragments are indicated by the * and ** symbols, respectively. See also Figure S1.

(C) Schematic representation of the two *Il4* alleles in KN2-KN2, KN2-WT, and KN2-V allelic reporter mice.

expressed gene *Rad50* (Figure 1A; Agarwal and Rao, 1998; Ansel et al., 2006; Wilson et al., 2009). Targeted deletion of selected *cis*-regulatory elements in mice demonstrated their nonredundant functions in regulating Th2 cell-type cytokine gene expression (Ansel et al., 2004; Koh et al., 2010; Lee et al., 2003; Loots et al., 2000; Mohrs et al., 2001; Solymar et al., 2002; Tanaka et al., 2006, 2011; Yagi et al., 2007).

We previously identified two putative distal enhancers located 3′ of the *Il4* gene, marked by cell type-specific DNase I hypersensitivity (hypersensitivity site V: HS V and HS $V_A$) (Figure 1A; Figure S1A available online). HS V is not accessible in naive T cells or differentiated Th1 cells, but becomes constitutively accessible in resting Th2 cells; it overlaps a highly conserved noncoding sequence (CNS2) in the *Il4* locus (Ansel et al., 2006). HS $V_A$ becomes accessible only upon activation of Th2 cells, and the corresponding sequence binds GATA3, STAT6, and NFAT (Agarwal et al., 2000). Combined deletion of a 3.7 kb region spanning both HS V and HS $V_A$ resulted in impaired IL-4 and IL-13 production in both Th2 cells and mast cells (Solymar et al., 2002). Confirming these findings, a similar strain of CNS2-deficient mice (Yagi et al., 2007), which bear a smaller deletion that disrupts HS V but also deletes about half of the sequence corresponding to HS $V_A$ (Figure S1A), including NFAT and GATA3 binding sequences (Agarwal et al., 2000), also showed impaired IL-4 production in NK T cells and T-CD4 T cells (Sofi et al., 2011; Yagi et al., 2007). Unfortunately, the functional impairment in cytokine production observed in HS V and $V_A$-deficient and in CNS2-deficient mice could not be unambiguously attributed to one or the other region, because the integrity of both putative regulatory regions was compromised.

There are compelling reasons to examine the function of the HS V (CNS2) region in isolation. The interesting features of this region include (1) constitutive accessibility in Th2 cells (Agarwal and Rao, 1998); (2) DNA hypomethylation in naive T cells (Lee et al., 2002); (3) maintained DNA hypomethylation during Th2 cell differentiation but increased DNA methylation during Th1 cell differentiation (Lee et al., 2002); and (4) binding of a number of important transcriptional regulators—including STAT6, STAT5, GATA3, Notch, RBP-Jκ, ATP-dependent chromatin remodeler BRG-1, chromatin looping factor SATB1, and histone methyl transferase MLL—to the HS V region in a Th2 cell-preferential manner (Cai et al., 2006; Liao et al., 2008; Tanaka et al., 2006, 2011; Wei et al., 2010; Wurster and Pazin, 2008; Yamashita et al., 2006).

To address these issues, we generated mice bearing a precise deletion of the HS V (CNS2) region. An unexpected finding in the HS V-deficient (ΔV) mice was the complete abrogation of IgE production despite only mild reduction in type 2 cellular responses in affected tissues. To determine whether this dichotomous response was due to the differential requirement for HS V by the cell types that produce IL-4, we made use of allelic IL-4 reporter mice, which allowed us to track IL-4-producing cells in vivo. We show that Tfh cells critically depend on HS V for IL-4 production. In contrast, effector Th2 cells, basophils, and eosinophils were far less dependent on HS V.

## RESULTS

### Deletion of HS V Impairs *Il4* Transcription

To examine the function of HS V in regulation of Th2 cell-type cytokine genes, we generated mice with a specific deletion of HS V that did not disrupt the adjacent enhancer, HS $V_A$ (Figures 1A and S1). DNase I hypersensitivity analysis of in-vitro-polarized HS V-deficient (ΔV) Th2 cells confirmed selective loss of HS V; importantly, other hypersensitivity sites that mark *cis*-regulatory elements remained intact, including the activation-inducible site HS $V_A$ (Figure 1B). Unlike Th2 cells from mice

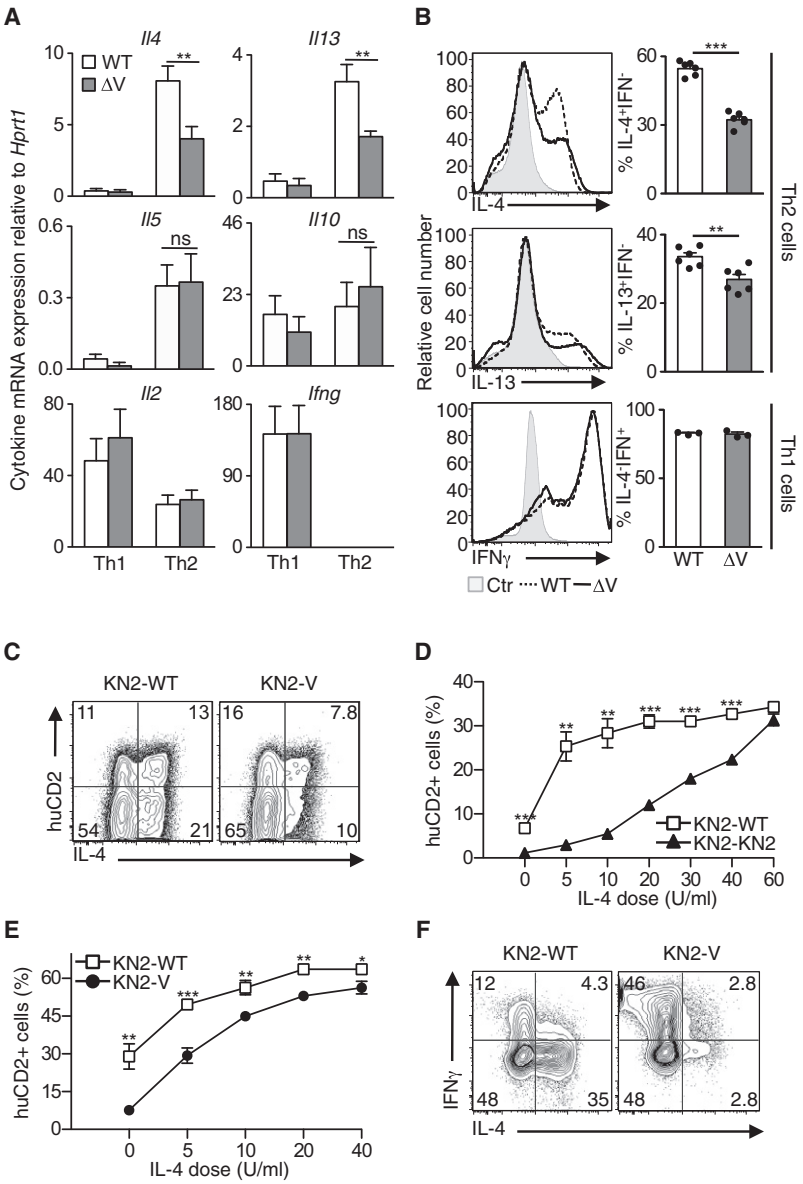


**Figure 2. Cytokine Gene Expression Profile of HS V-Deficient CD4⁺ T Cells**

(A) *Hprt1*-normalized cytokine mRNA abundance in CD4⁺ T cells differentiated in vitro under Th2 and Th1 cell polarizing conditions for 1 week and stimulated with PMA and ionomycin for 4 hr. Bars display average and error bars indicate standard error of mean.

(B) Histograms show intracellular cytokine staining of cells described in (A).

(C) Contour plots show IL-4 and huCD2 staining in Th2 cells generated in vitro (A).

(D and E) CD4⁺ T cells from the indicated mice were differentiated in vitro under submaximal Th2 cell polarizing conditions. Graphs show the percentage huCD2⁺ cells in relation to the concentration of exogenous IL-4 added to the culture. See also Figure S2A.

(F) Contour plot shows intracellular cytokine staining of restimulated CD4⁺ T cells from KN2-WT and KN2-V mice cultured with 11 U/ml of IL-4. See also Figure S2B.

n.s., no statistically significant difference ($p > 0.05$), $^{**}p < 0.01$, $^{***}p < 0.001$ with Student's t test. See also Table S2.

with the combined HS V and $V_A$ deletion, which show diminished transcription of all the linked Th2 cell-type cytokine genes (*Il4, Il13*, and *Il5*) (Solymar et al., 2002), restimulated ΔV Th2 cells showed a nearly 50% reduction in the expression of *Il4* and *Il13* mRNA, but no significant change ($p > 0.05$) in *Il5* and *Il10* (Figure 2A). Compared to wild-type (WT) Th2 cells, the frequency of restimulated ΔV Th2 cells producing IL-4 (mean ± SEM, 55% ± 1.3% versus 32% ± 1.3%) and IL-13 (mean ± SEM, 34% ± 1.2% versus 27% ± 1.5%) protein was also reduced by 40% and 20%, respectively, (Figure 2B). As expected, the cytokine profile of ΔV Th1 cells was similar to that of WT Th1 cells (Figures 2A and 2B).

As a major product of Th2 cells that is also a potent inducer of Th2 cell differentiation, IL-4 is the key element of a positive-feedback mechanism that polarizes Th2 cell responses both in vitro and in vivo. To assess the requirement for HS V under conditions where this positive feedback was minimal, we generated heterozygous allelic reporter mice in which one *Il4* allele derives from KN2 reporter mice (Mohrs et al., 2005) and the second is wild-type or bears the HS V deletion (designated KN2-WT and KN2-V respectively; Figure 1C). In the KN2 allele, a *CD2* gene cassette replaces the first two exons of *Il4*; thus, IL-4 protein is not produced but *Il4* transcription is faithfully reported as surface expression of human CD2 (huCD2) (Mohrs et al., 2005). Th2 cell cultures from both allelic reporter mice contained equal numbers of huCD2⁺ cells, indicating comparable Th2 cell polarization; among these huCD2⁺ IL4-competent cells, however, the frequency of IL-4 production was reduced in KN2-V T cells compared to KN2-WT cells, confirming a direct *cis*-regulatory role for HS V in the control of *Il4* activity in Th2 cells (Figure 2C).

To assess the magnitude of positive feedback through IL-4, we compared huCD2 expression in KN2-WT and KN2-KN2 T cells, which do and do not produce IL-4, respectively. As expected, endogenous IL-4 produced from the functional IL-4 allele in KN2-WT T cells strongly potentiated Th2 cell polarization when the cells were differentiated under suboptimal Th2 cell conditions, with limiting amounts of exogenous IL-4 provided in culture (Figures 2D and S2A). Similarly, a 2- to 3-fold lower dose of exogenous IL-4 was necessary to induce huCD2 expression in KN2-WT T cells (which produce their own endogenous IL-4), compared to KN2-V T cells (which lack HS V in the functional IL-4 allele) (Figures 2E and S2A). At a low concentration of exogenous IL-4 (11 U/ml), the cytokine profile indicated a very strong dependence for HS V in Th2 cell polarization (Figures 2F and S2B). These results suggested that the deletion of HS V was likely to have pronounced effects on in vivo responses in which IL-4 feedback is important.

## In Vivo Type 2 Responses in HS V-Deficient Mice

To determine the consequences of HS V deficiency in vivo, we used a mouse model of allergic airway disease. Airway

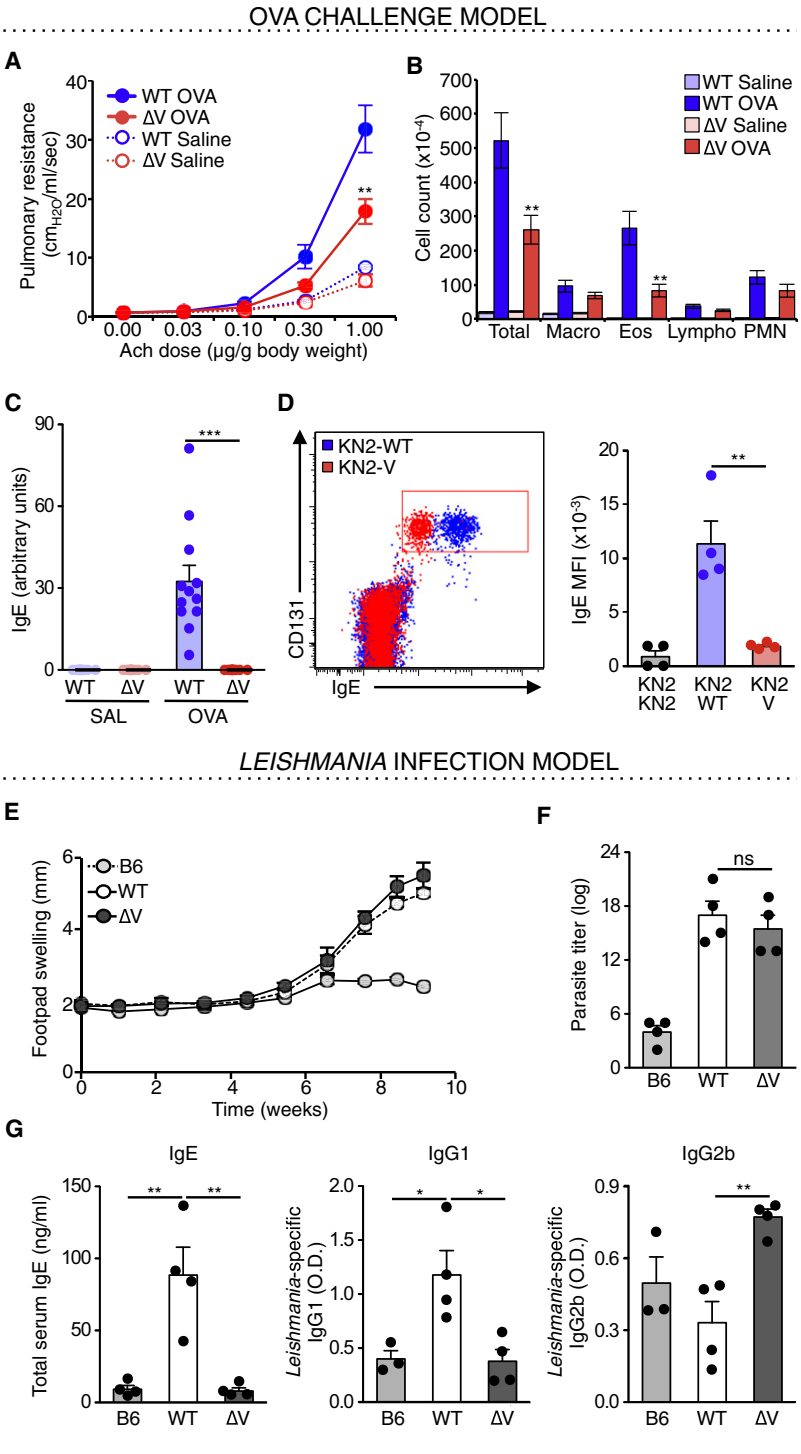


**Figure 3. In Vivo Type 2 Responses in HS V-Deficient Mice**

Cohorts of wild-type (WT) and HS V-deficient (ΔV) BALB/c mice were subjected to the ovalbumin (OVA) model of allergic airway disease.

(A) Airway hyperresponsiveness after saline (n = 10) and OVA challenge (n = 12) was measured as increasing pulmonary resistance in response to acetylcholine (ach). Data are shown as mean ± standard error mean (SEM).

(B) Total number and cellular composition of the leukocytes in bronchoalveolar lavage fluid (mean ± SEM); macrophages (Macro), eosinophils (Eos), lymphocytes (Lympho), and polymorphonuclear neutrophils (PMN).

(C) Serum OVA-specific IgE abundance measured by ELISA (n = 12). Average (bars) of data from individual mice (filled circles) are shown; error bars are SEM.

(D) Overlay of dot plots showing IgE and CD131 staining of live, singlet-gated, $CD45^+$ cells infiltrating the lungs of OVA-challenged KN2-WT and KN2-V mice. Graph at right shows mean fluorescence intensity (MFI) of IgE staining on basophils.

(E–G) Cohorts of four wild-type C57BL/6 (B6), wild-type BALB/c (WT), and HS V-deficient BALB/c (ΔV) mice were infected with *Leishmania major* promastigotes in the hind footpad.

(E) Footpad swelling over the course of infection.

(F) Footpad parasite burden (log titer) 64 days after infection.

(G) ELISA measurement of total serum IgE and *Leishmania* freeze-thaw antigen-specific IgG1 and IgG2b.

n.s., no statistically significant difference ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with Student's t test.

hyperresponsiveness (AHR) was reduced in OVA-challenged ΔV mice compared to WT controls (Figure 3A). Peribronchial and perivascular inflammatory infiltrates and mucus hypersecretion typical of allergic inflammation were preserved in ΔV mice (data not shown), but reduced numbers of eosinophils and lymphocytes were found in the bronchoalveolar lavage fluid (Figure 3B). Overall, therefore, the pathological type 2 cellular response in the lungs was partially diminished in ΔV mice.

Immunoglobulin (Ig) isotype switching to IgE, a hallmark of type 2 humoral immunity, is known to be critically dependent on IL-4 production (Finkelman et al., 1988; Reinhardt et al., 2009). Strikingly, the IgE response was completely abolished in ΔV mice (Figure 3C). Flow cytometric measurement of IgE bound to lung-infiltrating basophils confirmed an almost log-scale reduction in the amount of IgE present in ΔV mice (Figure 3D). Because basophil IgE staining in KN2-V mice was very similar to that seen in IL-4-deficient KN2-KN2 mice (Figure 3D), we conclude that HS V is critically required for IL-4 production by the cells that direct IgE responses in vivo.

To corroborate our findings on the effects of HS V deficiency in the asthma model in vivo, we utilized *Leishmania* infection. The magnitude of type 2 responses in this model inversely correlates with the capacity to clear parasites and resolve tissue inflammation (Mohrs et al., 2001). Thus, control C57BL/6 mice that primarily mount a type 1 response with abundant IFN-γ-producing Th1 cells effectively cleared the parasite. BALB/c mice, which mount a sustained type 2 response, showed a significant increase in the size of footpad lesions and parasite burden up to 10 weeks after infection (Figures 3E and 3F). We observed no difference between ΔV and WT BALB/c mice, suggesting that in this experimental

system, there was no diminution in type 2 tissue responses in the absence of HS V (Figures 3E and 3F). Nevertheless, the type 2 humoral response was again significantly affected in the ΔV mice as reflected by the complete absence of IgE and a reduction of *Leishmania*-specific IgG1, which is also partly dependent on IL-4 (Kopf et al., 1993); *Leishmania*-specific IgG2b production, which is IL-4 independent, was unaffected (Figure 3G).

Together these results support an important role for HS V in type 2 humoral immunity in vivo. However, the dichotomous effects of HS V deficiency on the different arms of type 2 immune responses, namely the complete absence of the IL-4-dependent humoral response with at most a partial reduction in type 2 tissue responses, indicated that the cell types driving these responses were differentially affected by HS V deficiency.

## HS V-Deficient Tfh Cells Develop Normally but Fail to Produce IL-4

Previous studies with KN2 mice showed that the vast majority of huCD2$^+$ IL-4-competent cells in the lymph nodes are CXCR5$^+$ PD-1$^{hi}$ Tfh cells that reside in germinal centers and the follicular mantle zone (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). These Tfh cells, or their precursors generated early in immune responses, are the source of IL-4 and other signals that act on B cells to induce IgE and IgG1 production (King and Mohrs, 2009; Reinhardt et al., 2009; Zaretsky et al., 2009). The KN2 allele does not encode IL-4 protein but marks cells that are competent to make IL-4. Therefore, the allelic reporter mice allowed us to ask whether Tfh cells and other in-vivo-generated huCD2$^+$ IL-4-competent cells require the HS V *cis*-regulatory region for IL-4 production from the other allele.

In the asthma model, we observed a significant induction (3%–4%) of huCD2$^+$ T cells in the draining parathymic lymph nodes, but not in the nondraining inguinal nodes (0.1%–0.3%), 25 days after OVA immunization (Figure 4A, compare top and second panels). Consistent with previous reports (King and Mohrs, 2009; Reinhardt et al., 2009), equal numbers of huCD2$^+$ IL-4-competent cells and CXCR5$^+$PD-1$^{hi}$ Tfh cells were observed in the parathymic lymph nodes of KN2-WT, KN2-V, and KN2-KN2 mice (Figure 4A, second and third panels; quantified in the bar graphs to the right), indicating that IL-4 (lacking in KN2-KN2 mice) and the enhancer activity of HS V (lacking in KN2-V compared to KN2-WT mice) are both dispensable for the generation of Tfh cells in vivo. CXCR5$^+$PD-1$^{hi}$ Tfh cells were predominantly huCD2$^+$ (Figure 4A, third panel, inset histograms), implying that the majority of Tfh cells transcribed the KN2 reporter allele in vivo. Conversely, essentially all huCD2$^+$ cells in the draining lymph nodes also expressed CXCR5 and PD-1 (Figure 4A, bottom panel), confirming that the vast majority of IL-4-competent T cells in this location are indeed Tfh cells under these experimental conditions.

The ability of these huCD2$^+$ cells to produce cytokines from the wild-type or HS V-deficient *Il4* allele was assessed ex vivo by intracellular staining and flow cytometric analysis of IL-4 production after stimulation with PMA and ionomycin for 4 hr. We prevented surface expression of any new huCD2 by inhibiting protein transport with brefeldin A during the entire time of in vitro stimulation, allowing us to focus on cells that were already expressing huCD2 in vivo. As expected of Tfh cells (Reinhardt et al., 2009), the huCD2$^+$CD4$^+$ cells from KN2-WT mice predominantly made IL-4 and not IL-13; strikingly, however, huCD2$^+$ CD4$^+$ cells from KN2-V mice almost completely failed to make any IL-4 even under these supraphysiological stimulation conditions (Figure 4B, top panel). As expected, similar results were observed when we restricted our analysis to CXCR5$^+$PD-1$^{hi}$ Tfh cells (Figure 4B, bottom panel). Thus, in Tfh cells exhibiting normal expression of huCD2 from the KN2 *Il4* reporter allele, the HS V-deficient allele is completely unable to support IL-4 production, again indicating a critical *cis*-acting requirement for HS V for *Il4* expression in Tfh cells.

These findings were confirmed in *Leishmania* infection. When CD4$^+$ T cells taken from the draining popliteal lymph nodes 10 weeks after infection were restimulated in vitro, we observed a significant reduction in the number of IL-4-producing cells in ΔV mice compared to WT BALB/c mice (Figure 4C). IL-4 production was also affected in vivo, as evidenced by the near absence of *Il4* mRNA in freshly isolated lymph node cells from ΔV mice (Figure 4D).

We next utilized an acute LCMV (lymphocytic choriomeningitis virus) infection model that allowed us to generate relatively larger numbers of IL-4-producing Tfh cells for undertaking detailed mRNA and chromatin analyses (Yusuf et al., 2010). Consistent with our findings in the OVA model, comparable numbers of CXCR5$^+$PD-1$^{hi}$ Tfh cells and germinal center B cells were observed in the lymph nodes and spleen of both WT and ΔV mice (Figures 4E and 4F). Expression of *Bcl6*, *Blimp1*, *ICOS*, SLAM-associated protein (SAP, encoded by *Sh2d1a*), and *Il21* mRNA in FACS-sorted CXCR5$^+$PD-1$^{hi}$ Tfh cells freshly isolated from lymph nodes and spleen were not significantly different between WT and ΔV mice (Figure 4G), further confirming that the enhancer activity of HS V is dispensable for the generation of Tfh cells in vivo. However, IL-4 production was significantly reduced in vivo, as evidenced by the near absence of *Il4* mRNA in the FACS-sorted CXCR5$^+$PD-1$^{hi}$ Tfh cells from ΔV mice (Figure 4H). We conclude that ΔV Tfh cells are phenotypically normal but have an isolated defect in *Il4* transcription.

## Early IL-4 Production by Lymph Node T Cells Is Dependent on HS V

Because IgE is produced at early times after sensitization with OVA, we measured OVA-specific IgE, IgG1, and IgG2b at days 9 and 14 after primary immunization with OVA (Figure 5A). Comparison of KN2-WT and KN2-KN2 mice (which produce and lack IL-4, respectively) emphasized the requirement for IL-4 in IgE and IgG1 responses (Figure 5A, left and middle panels). KN2-V mice resembled KN2-KN2 mice in that they failed to generate an IgE response and produced markedly reduced quantities of OVA-specific IgG1 (Figure 5A, left and middle panels). The IL-4-independent IgG2b response was comparable in the three groups of mice (Figure 5A, right panel).

We also assessed cytokine production by huCD2$^+$CD4$^+$ Tfh cells after ex vivo stimulation on days 4 and 7 after intraperitoneal OVA-alum immunization. As before (Figure 4B), huCD2$^+$ IL-4-competent parathymic lymph node T cells from KN2-V mice failed to produce IL-4 (Figure 5B). We obtained similar results when T cells in the lung-draining mediastinal nodes were analyzed after inhaled OVA challenge in the asthma model (Figure 5C). Together, these findings support a key *cis*-regulatory

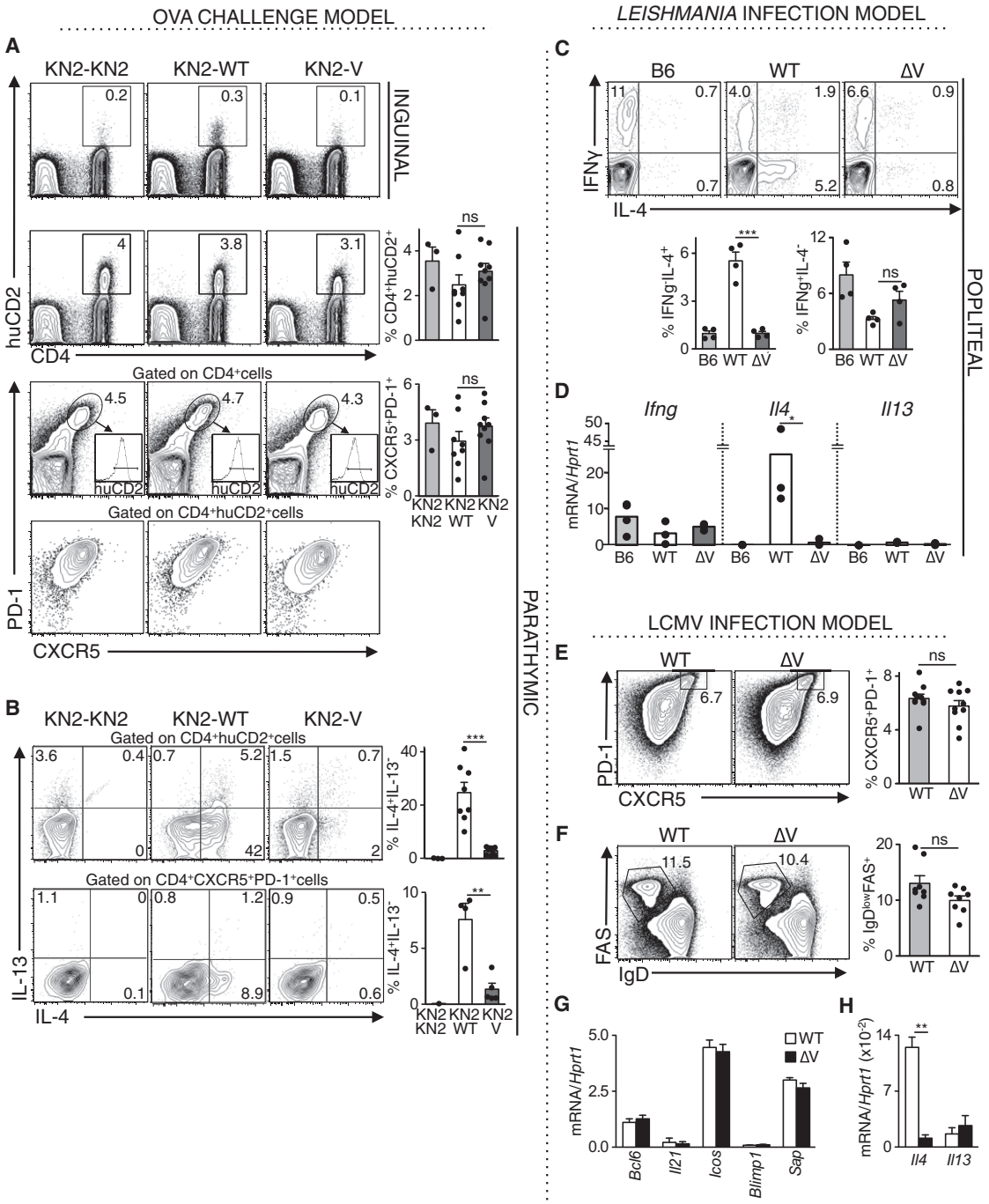


**Figure 4. HS V-Deficient Tfh Cells Fail to Produce IL-4**

(A) Cohorts of KN2-KN2, KN2-WT, and KN2-V BALB/c mice were subjected to the ovalbumin (OVA) model of allergic airway disease. Contour plots for CD4 and huCD2 show all live and singlet-gated lymph node cells. Numbers indicate percentage of cells that are huCD2$^+$CD4$^+$. Contour plots for CXCR5 and PD-1 show all CD4$^+$CD8$^-$B220$^-$ cells (third row) or only huCD2$^+$CD4$^+$CD8$^-$B220$^-$ cells (fourth row). Numbers indicate percentage of CXCR5$^+$PD-1$^+$ cells; inset histograms show huCD2 staining of these cells. Bars represent the average and filled circles represent data from individual mice; error bars are standard error mean (SEM).

(B) Contour plots show intracellular staining of cytokines in restimulated huCD2$^+$CD4$^+$ (top) and CXCR5$^+$PD-1$^+$ (bottom) lymph node cells described in (A).

(C) Cohorts of wild-type C57BL/6 (B6), wild-type BALB/c (WT), and HS V-deficient BALB/c mice (ΔV) were infected with *L. major*. Ten weeks later, restimulated T cells in the draining popliteal lymph nodes were analyzed by flow cytometry. Contour plots show intracellular staining of cytokines in size-gated CD4$^+$CD8$^-$B220$^-$ cells. Data are summarized in graphs shown below.

(D) *Hprt1*-normalized *Ifng*, *Il4*, and *Il13* mRNA abundance in unstimulated popliteal lymph node cells from *L. major*-infected mice.

(E and F) Cohorts of WT and HS V-deficient C57BL/6 mice (n = 4–10 per experiment) were infected with LCMV. Two weeks later, CD4$^+$ T cells and B cells isolated from lymph nodes and spleen were analyzed by flow cytometry.

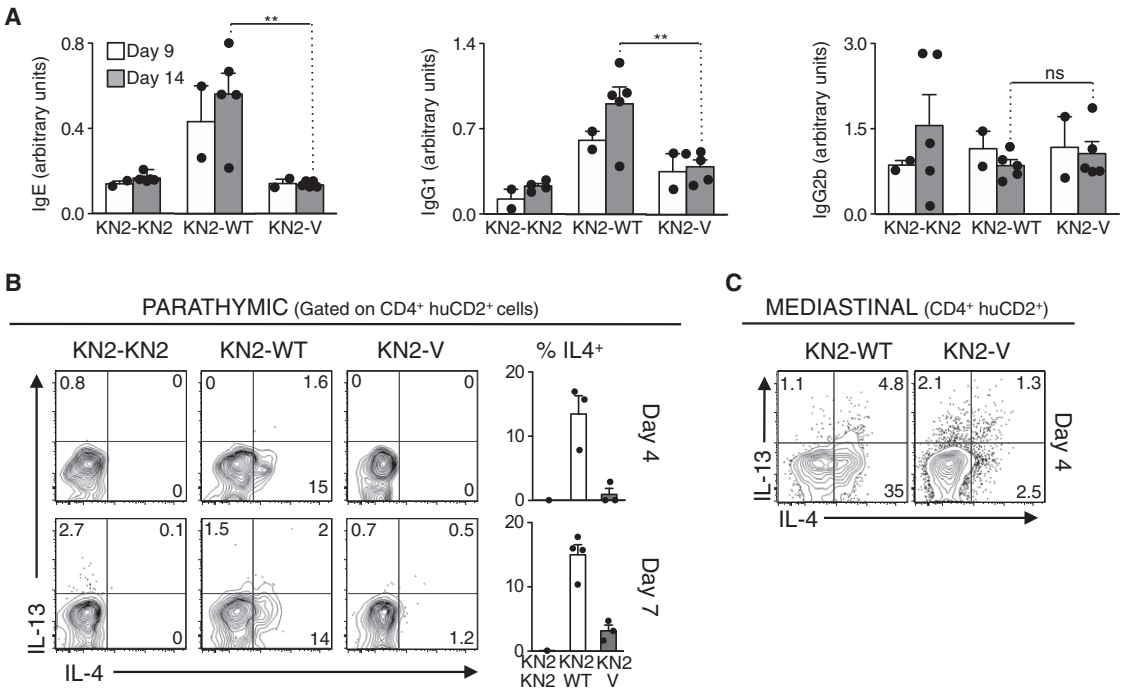


**Figure 5. HS V-Deficient Lymph Node T Cells Fail to Produce IL-4 Early in the Primary Immune Response**

Cohorts of KN2-KN2, KN2-WT, and KN2-V BALB/c mice were sensitized to ovalbumin by intraperitoneal immunization.

(A) ELISA measurement of ovalbumin-specific IgE, IgG1, and IgG2b levels in serum. Bars represent the average; error bars are SEM; circles represent data from each mouse.

(B) On days 4 and 7, the draining parathymic lymph node cells were stimulated in vitro with PMA, ionomycin, and brefeldin A for 4 hr and analyzed by flow cytometry. Contour plots show intracellular staining of cytokines in size-gated huCD2$^+$CD4$^+$CD8$^-$B220$^-$ cells. Data are summarized in the bar graphs to the right.

(C) Three weeks after sensitization, mice were intranasally challenged with ovalbumin for 3 consecutive days, and T cells in the lung-draining mediastinal lymph nodes were analyzed as described in (B).

function for HS V in T cells producing IL-4 very early in the immune response and suggest that Tfh cells and their early precursors share similar requirements for *Il4* regulation.

## Type 2 Tissue Responses in the Lungs of HS V-Deficient Mice

Given that tissue inflammation and AHR were less severely affected than humoral immune responses in ΔV mice, we hypothesized that the cell types and cytokines that drive type 2 tissue responses are less dependent than Tfh cells on HS V. To test this hypothesis, we again employed *Il4* allelic reporter mice, in this case focusing on CD4$^+$ T cells, basophils, and eosinophils as the three major IL-4-producing cell types in allergic lung inflammation (Mohrs et al., 2005; Voehringer et al., 2006).

In contrast to Tfh cells, lung-infiltrating Th2 cells produced IL-13 when restimulated ex vivo (Figure 6A, top left panel). Comparing KN2-KN2, KN2-WT, and KN2-V mice, a similar proportion of CD4$^+$ T cells expressed huCD2 from the *Il4* KN2 reporter allele, which has an intact HS V (Figure 6A, right top panel). Thus IL-4, which is absent in KN2-KN2 mice, is dispensable for the generation of CD4$^+$ Th2 cells in this experimental system. However, the fraction of T cells able to express IL-4 protein from the other allele upon restimulation was reduced in KN2-V mice compared to KN2-WT mice (Figure 6A, top panels). This effect could not be explained by feedback from reduced IL-4 production in vivo, because it was apparent even when we restricted our analysis to huCD2$^+$ T cells or to cells that produced IL-13 upon restimulation (Figure 6B, top panel). Thus, HS V enhances *Il4* expression *in cis* in Th2 cells in vivo, consistent with our in vitro findings.

In contrast to Th2 cells, lung-infiltrating basophils produced IL-4 in an entirely HS V-independent fashion upon in vitro stimulation with PMA and ionomycin (Figure 6A, bottom panels). Moreover, in vitro, all huCD2$^+$ basophils produced IL-4 and IL-13, confirming that HS V is not essential for IL-4 production by basophils (Figure 6B, bottom panel). We were unable to measure IL-4 protein production by eosinophils, but FACS-sorted eosinophils

(E) Contour plots for CXCR5 and PD-1 show size-gated CD4$^+$CD44$^{hi}$CD62L$^-$CD8$^-$B220$^-$ cells. Numbers indicate percentage of CXCR5$^+$PD-1$^+$ cells.

(F) Contour plots for IgD and FAS show size-gated CD19$^+$CD4$^-$CD8$^-$ cells. Numbers indicate percentage of IgD$^{lo}$FAS$^+$ cells. Summary of data from four independent experiments are shown in graphs at right.

(G and H) *Hprt1*-normalized *Bcl6*, *Blimp1*, *ICOS*, SLAM-associated protein (SAP, encoded by *Sh2d1a*), *Il21, Il4,* and *Il13* mRNA in FACS-sorted pure populations of CXCR5$^+$PD-1$^{hi}$ Tfh cells, freshly isolated from lymph node cells and spleen of mice described in (E).

n.s., no statistically significant difference ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with Student's t test.

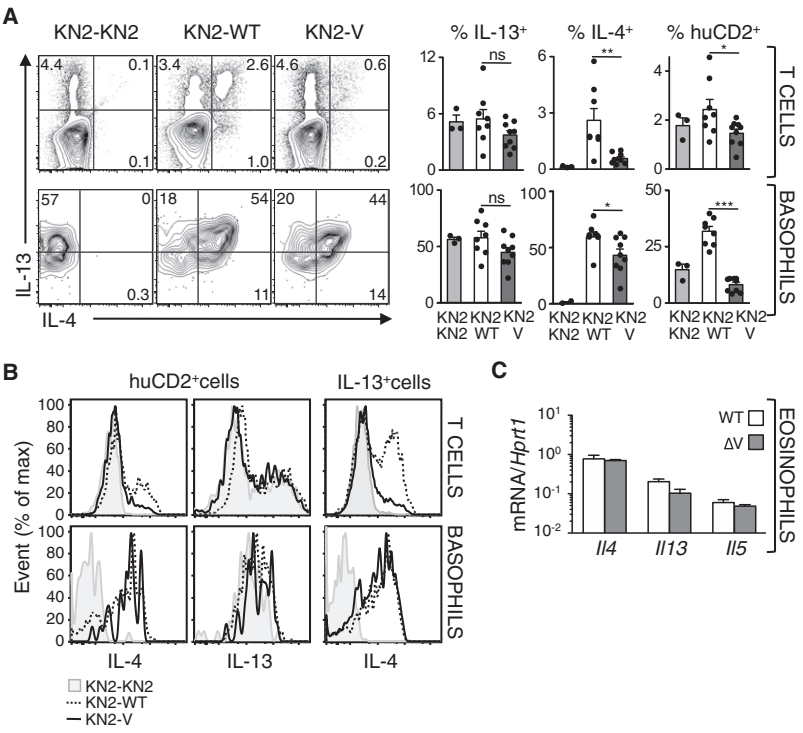


**Figure 6. HS V Function in IL-4-Producing Cells Infiltrating the Lungs in a Murine Model of Asthma**

Cohorts of KN2-KN2, KN2-WT, and KN2-V BALB/c mice were sensitized and challenged with ovalbumin to induce allergic airway inflammation. Lung-infiltrating cells were analyzed by flow cytometry after in vitro stimulation with PMA and ionomycin. See also Table S1.

(A) Contour plots show intracellular staining of cytokines in $CD4^+CD3^+CD8^-B220^-$ cells (T cells) and $CD45^+IgE^+CD49b^+CD3^-MHCII^-$ cells (basophils). See also Figure S4. Graphs at right show compiled data for IL-4, IL-13, and huCD2 expression. Bars represent the average; error bars are SEM; filled circles represent data from each mouse.

(B) Histograms of IL-4 and IL-13 staining in $huCD2^+$ T cells and basophils, and IL-4 staining in $IL\text{-}13^+$ cells as indicated.

(C) *Hprt1*-normalized cytokine mRNA abundance in FACS-sorted $CD45^+CD3^-CD11b^+MHCII^-CD11c^-SiglecF^+$ cells (eosinophils). See also Figure S4.

n.s., no statistically significant difference ($p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ with Student's t test.

from the lungs of OVA-challenged WT and ΔV mice expressed *Il4* mRNA at comparable levels (Figure 6C).

Notably, the proportion of basophils expressing huCD2 in vivo was reduced in KN2-KN2 mice compared with KN2-WT mice (Figure 6A, bottom right panel), indicating that IL-4 is important for basophil expression of *Il4* in vivo. A similar reduction in $huCD2^+$ basophils was also observed in KN2-V mice (Figure 6A, bottom right panel), suggesting that the source of IL-4 that affects basophil numbers in vivo is strongly HS V dependent. We speculate that because Tfh cells in KN2-KN2 and KN2-V mice cannot produce IL-4 (Figure 4B), the consequent drastic decrease in IgE (Figures 3 and 5) deprives basophils of their ability to use antigen-specific IgE to respond to OVA challenge in vivo.

In summary, unlike the Tfh cells that direct type 2 humoral responses and are strongly dependent on HS V, the cells and cytokines involved in type 2 tissue responses are less dependent, or only indirectly dependent, on HS V for IL-4 production.

## HS V Affects Chromatin Accessibility and NFAT Binding to the *Il4* Promoter

To investigate the mechanism by which HS V deficiency affected *Il4* transcription in T cells, we first tested whether loss of HS V affected early transcription of the *Il4* gene by naive T cells. Remarkably, naive T cells lacking the HS V (CNS2) region were completely unable to produce *Il4* transcripts after ex vivo stimulation (Figure 7A). These findings are reminiscent of our observations in Tfh cells and their early precursors (Figures 4B, 4F, 5B, and 5C) and suggest that all of these cells share similar *cis*-regulatory requirements for *Il4* transcription. In a similar time course assay, in-vitro-differentiated Th2 cells showed only a 50% reduction in *Il4* transcription (Figure 7B).

*Il4* transcription by naive T cells is strongly dependent on the NFAT-calcineurin pathway, as judged by sensitivity to the calcineurin inhibitor cyclosporin A (CsA) (Ansel et al., 2004) and by the binding of NFAT proteins to the *Il4* promoter (Ansel et al., 2004). Chromatin immunoprecipitation (ChIP) assays performed after brief (45 min) stimulation showed that binding of the transcription factor NFAT1, the predominant NFAT family member in naive T cells (Macián et al., 2002), was severely reduced in ΔV naive T cells as well as polarized Th2 cell populations (Figures 7B, S3A, and S3B). Together, these results suggest an important role for a distal *cis*-regulatory region, HS V (CNS2), in facilitating NFAT1 binding to the *Il4* promoter upon T cell activation.

The diminished binding of NFAT1 to the *Il4* promoter in ΔV T cells prompted us to examine the effect of HS V deficiency on chromatin structure in the *Il4* locus in naive T cells and differentiated Th2 cells (Figures 7C and 7D). Because *Il4* transcription was selectively affected in T cells, with minimal or no effect on *Il13* and *Il5* expression, respectively, we focused on known *cis*-regulatory elements near the *Il4* gene: the *Il4* promoter; the 3′ *Il4* silencer, HS IV (Ansel et al., 2004); HS $V_A$ (Agarwal et al., 2000; Agarwal and Rao, 1998); and HS V (CNS2) itself. We limited our analysis to two histone modifications whose association with gene expression has been thoroughly documented: histone-3 lysine-4 dimethylation (H3K4me2), a "permissive" modification found at enhancers and promoters whose presence correlates with increased chromatin accessibility to *trans*-factors (Birney et al., 2007); and H3K27me3, a "repressive" mark (Bernstein et al., 2006; Wei et al., 2009).

Of the four regions tested, HS V (CNS2) displayed by far the highest enrichment for H3K4me2 in naive T cells and Tfh cells (Figure 7C, black bars; Baguet and Bix, 2004). H3K4me2 was retained at HS V during Th2 cell differentiation and increased at the *Il4* promoter and HS $V_A$ (Figure 7C, black bars). HS V deficiency did not influence H3K4me2 levels in naive T cells and Tfh cells (Figure 7C, compare black and gray bars). In differentiated

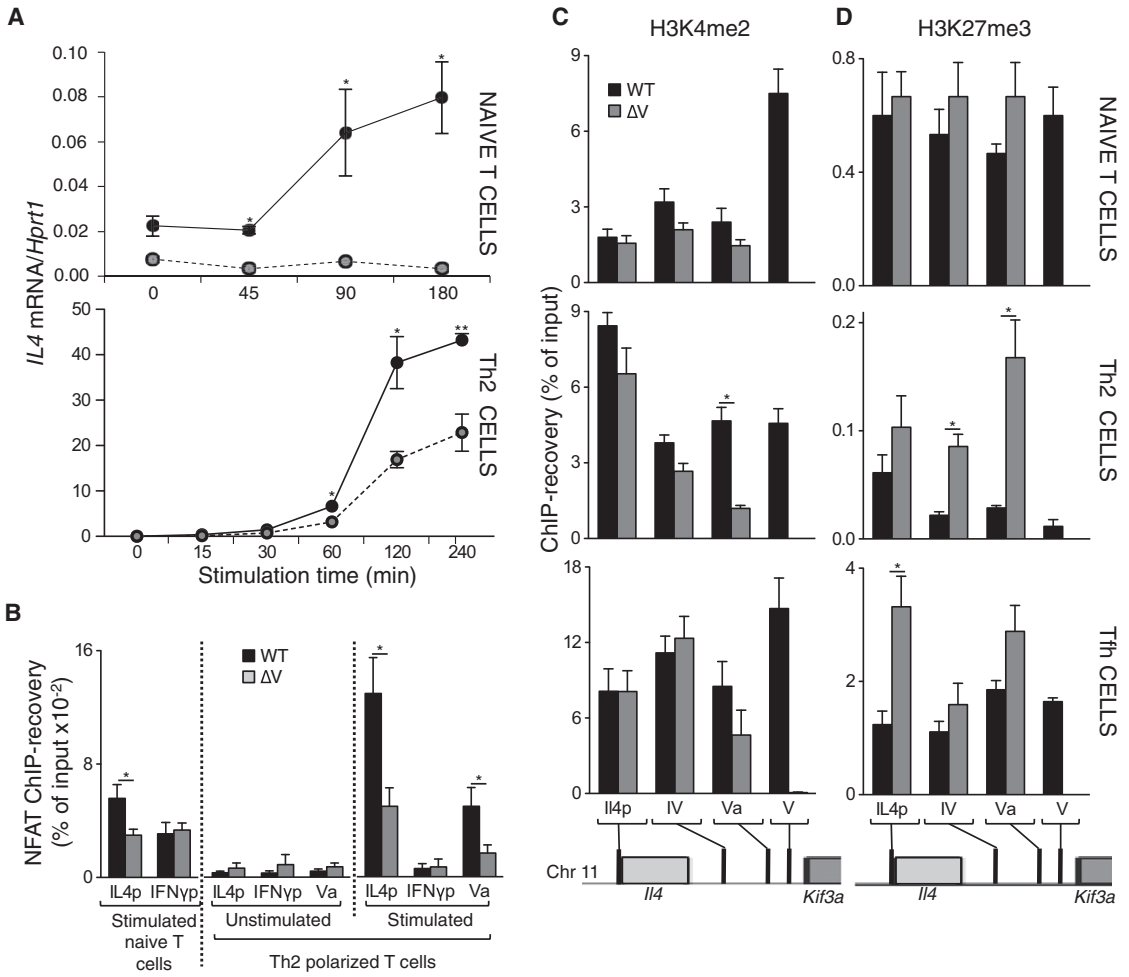


**Figure 7. Chromatin Accessibility and NFAT Binding in the Th2 Cell Locus of HS V-Deficient T Cells**

(A) *Hprt1*-normalized *Il4* mRNA abundance in wild-type (WT) and HS V-deficient (ΔV) naive T cells and Th2 cells (derived in vitro) stimulated with CD3 and CD28 antibodies and PMA and ionomycin, respectively, for the indicated times. Dots display average and error bars indicate standard error of mean (SEM).

(B) ChIP-PCR analysis: real-time PCR quantification of *Il4* promoter (IL-4p), HS $V_A$, and *Ifng* promoter (IFNγp) sequences after ChIP with antibody to NFAT in WT and ΔV Th2 cells and naive T cells, either left unstimulated or stimulated for 45 min with PMA and ionomycin. Data are expressed as the normalized percentage of input DNA recovered and represent mean and SEM of at least three independent ChIP experiments. Data were normalized to the mean ChIP recovery of all experiments. Raw data from independent experiments are shown in Figures S3A and S3B.

(C and D) Real-time PCR quantification of *Il4* promoter (IL-4p), HS IV, HS $V_A$, and HS V sequences after anti-H3K4me2 (C) and anti-H3K27me3 (D) ChIP of chromatin extracts obtained from resting $CD4^+$ naive T cells, Th2 cells derived in vitro, and Tfh cells derived in vivo from wild-type (WT) and HS V-deficient (ΔV) mice. Data are expressed as the percentage of input DNA recovered and represent mean and SEM of at least three independent ChIP experiments. Data from anti-H3K27me3 ChIP of Tfh cells were normalized and raw data from independent experiments are shown in Figure S3C. Real-time PCR quantification of control sequences is shown in Figure S3E. See also Figure S3D and Table S2.

ΔV Th2 cells, H3K4me2 levels remained lower than WT at HS $V_A$ (Figure 7C).

In naive T cells, the *Il4* promoter and *cis*-regulatory regions were also substantially enriched for H3K27me3 (Figure 7D, top panel). Notably, however, Th2 cell differentiation led to a striking loss of the "repressive" H3K27me3 modification at all four tested regions of the *Il4* gene (Koyanagi et al., 2005) (Figure 7D, compare black bars in top and middle panels), and Th2 cells from ΔV mice incompletely erased the repressive H3K27me3 mark, especially at HS $V_A$ (Figure 7D, middle panel, compare black and gray bars). Defective erasure of repressive H3K27me3 marks was also observed in ΔV Tfh cells, especially at the *Il4* promoter (Figure 7D, bottom panel and Figure S3C). Together, these data indicate that HS V (CNS2) is an important player in the chromatin-remodeling events that normally establish an accessible conformation across the *Il4* locus in Th2 cells and Tfh cells.

## DISCUSSION

We have performed a detailed analysis of mice bearing a precise deletion of HS V (CNS2). Our results show unambiguously that this conserved *cis*-regulatory element has an important and nonredundant function in enhancing *Il4* transcription. In

two in vivo models, HS V-deficient mice exhibited cell type-specific defects in *Il4* expression that manifested in surprisingly dichotomous effects on type 2 immune responses in vivo—a profound reduction of type 2 humoral immunity with total abrogation of IgE production, in the face of only mildly attenuated or unaffected type 2 tissue inflammatory responses.

We used allelic IL-4 reporter mice to uncover differential requirements for HS V among the cell types that drive these responses. We show that Tfh cells, lymph node T cells that make IL-4 early in the primary immune response, and even naive T cells stimulated ex vivo are strikingly dependent on HS V for *Il4* expression. These findings suggest that similar signals and transcription factors are responsible for *Il4* expression in all of these lymph node-resident T cells and probably explain the total abrogation of IgE production and sharply reduced IgG1 responses observed in HS V-deficient mice.

In contrast, Th2 cells derived in vivo or in vitro were only partially dependent on HS V for *Il4* expression. IL-4 signaling induces nuclear translocation of STAT6, which is a direct transactivator of both *Il4* and *Gata3.* GATA3, in turn, binds to its own promoter and several *cis*-regulatory sites in the Th2 cell-type cytokine locus, forming a feedforward positive-feedback loop that drives Th2 cell differentiation and the production of IL-13 and IL-5 (Zhu et al., 2010). Notably, HS V deficiency had minimal or no effect on IL-13 or IL-5 production by Th2 cells in vitro or in vivo. Thus, Th2 cells access an HS V-independent IL-4-driven positive-feedback loop to drive powerful inflammatory responses in tissues. HS V continues to function as a local enhancer of *Il4* in these cells but has only modest effects on their ability to marshal inflammatory responses.

Allergic inflammation in peripheral tissues also involves innate immune cells. A previous study detected HS V reporter transgenic activity in mast cells and basophils but found reduced IL-4 expression only in mast cells from mice lacking HS V and part of HS $V_A$ (Yagi et al., 2007). Our findings indicate that HS V affects basophil production of IL-4 in vivo but probably through an indirect mechanism. Given the importance of IgE receptor signaling in basophil activation, it is quite likely that their reduced IL-4 production in HS V-deficient mice reflects the lack of allergen-specific IgE. This effect may also contribute to the mild reduction in lung inflammation and AHR in HS V-deficient mice.

Notch intracellular domain and its binding partner RBP-Jκ bind to HS V and influence transcription of *Il4* in transgenic reporter assays (Amsen et al., 2004; Fang et al., 2007; Tanaka et al., 2006). Disruption of the Notch signaling pathway in mice leads to impaired humoral responses, as evidenced by sharply reduced IgE and IgG1 production and a significant reduction in IL-4 production by T cells in the draining lymph nodes and spleen, which presumably were Tfh cells (Amsen et al., 2007; Tanaka et al., 2006; Tu et al., 2005). The similarity of these findings with our observations in ΔV mice suggest that Notch may mediate its effects on *Il4* expression by Tfh cells more directly through HS V and implicates the Notch pathway as a critical regulator of Tfh cell function and humoral immunity. Further research is needed to determine the relative contribution of Notch and other *trans*-acting factors in HS V-dependent *Il4* expression in Tfh cells and how these factors mediate their effects on *Il4* locus chromatin structure and gene transcription.

To address the molecular mechanism by which HS V selectively affects *Il4* gene transcription, we examined the chromatin structure and remodeling events in the *Il4* locus during differentiation of naive cells into Th1, Th2 (in vitro), and Tfh (in vivo) cells. In naive T cells and Tfh cells, HS V displayed by far the highest enrichment for H3K4me2, suggesting increased chromatin accessibility at this site compared with other *cis*-regulatory elements in the locus (Baguet and Bix, 2004). HS V is also the only site of DNA demethylation between the *Il4* promoter and the distal *Kif3a* gene in naive T cells, suggesting a high degree of accessibility to *trans*-acting factors during early stages of T cell differentiation when other *cis*-elements in the locus may be relatively inaccessible (Lee et al., 2002). This feature, and the ability of the HS V (CNS2) region to enhance *Il4* transcription in a GATA3- and STAT6-independent manner, likely make HS V particularly critical for *Il4* transcription in naive T cells, Tfh cells, as well as in T cells that produce IL-4 early in the in vivo immune response.

H3K27me3, a repressive chromatin modification extensively present in the Th2 cell-type cytokine locus of naive T cells, is removed during Th2 cell differentiation, but maintained during Th1 cell differentiation (Figures 7D and S3A; Koyanagi et al., 2005). In ΔV Th2 cells, erasure of the H3K27me3 mark was incomplete across the locus, especially at HS $V_A$, where the mark was not erased at all. The failure to erase these marks was particularly pronounced in ΔV Tfh cells, correlating with the stringent requirement for HS V for *Il4* transcription in these cells. Further investigation is needed to determine whether HS V recruits histone demethylases to the locus and to probe the connection between removal of H3K27me3 marks and *Il4* promoter and enhancer accessibility for NFAT and other transcription factors that mediate *Il4* transcription.

In summary, our experiments have revealed a critical role for the distal *Il4* enhancer HS V in Tfh cell function and consequently type 2 humoral immunity. Mechanistically, HS V (CNS2) has an important role in shaping chromatin structure in differentiating T cells, as well as facilitating access of *trans*-acting factors such as NFAT to the *Il4* locus. Our data imply that Tfh cells and Th2 cells utilize distinct but overlapping molecular mechanisms to support *Il4* locus activity and may provide insight for more targeted strategies to block pathology in allergic diseases.

## EXPERIMENTAL PROCEDURES

### Mice

Mice were used in accordance with protocols approved by the animal care and use committees of the CBR Institute for Biomedical Research, Harvard Medical School, UCSF, and LIAI. ΔV mice were generated with standard gene-targeting techniques (details in Supplemental Information).

### T Cell Differentiation, FACS Analysis, and Quantification of Cytokine Messenger RNA Expression

Purification of $CD4^+$ T cells from spleen and lymph nodes, in vitro induction of Th1 and Th2 cell differentiation, and restimulation for flow cytometric analysis of intracellular cytokine staining and messenger RNA expression levels were performed as described previously (Ansel et al., 2004) (details in Supplemental Information). In brief, purified $CD4^+$ T cells were stimulated with hamster anti-mouse CD3 (clone 2C11, 0.25 μg/ml) and hamster anti-mouse CD28 (clone 37.51, 1 μg/ml) on plates coated with goat anti-hamster IgG (MP Biomedicals) for 48–60 hr under Th1 (IL-12 and anti-IL-4) and Th2 (IL-4, anti-IFN-γ, and anti-IL-12) cell or nonpolarizing conditions. After 2–3 days, cells were removed

from the plates and expanded in media with 20 U/ml of recombinant human IL-2 (National Cancer Institute) and analyzed on day 6. For short-term stimulation, $5 \times 10^6$ naive T cells were resuspended in media containing 0.5 μg/ml anti-CD3 and 1 μg/ml anti-CD28 and mixed with $2.5 \times 10^7$ latex beads (5 μm diameter; Interfacial Dynamics Corporation) coated with goat anti-hamster IgG. Unstimulated controls were cultured with beads but without anti-CD3 and anti-CD28 and were similar to cells held on ice.

### Experimental Allergic Lung Disease Model and Antibody Production

Mice were immunized on days 1, 7, and 14 by intraperitoneal (i.p.) injection of 50 μg OVA (Grade V; Sigma Aldrich)/1 mg alum (Thermo Scientific) emulsion, followed by intranasal challenge with saline (control) or 100 μg OVA on days 21, 22, and 23 as described (Kuperman et al., 2002). On day 24, measurement of airway resistance and BAL fluid total and differential cell counts were performed as described (Kuperman et al., 2002). Primary immune response was induced by a single i.p. injection of 50 μg OVA/1 mg alum emulsion, and serum samples were obtained at different time points for measuring OVA-specific IgE, IgG1, and IgG2b antibodies by ELISA. Isolation and flow cytometric analysis of immune cells present in lungs and lymph nodes are described in the Supplemental Information.

### *Leishmania* Infection Model

Amastigotes were serially passaged in the footpads of BALB/c mice to maintain *Leishmania major* LV39. Four mice per group were infected in the right hind footpad with $1 \times 10^6$ stationary-phase promastigotes. Lesion size was measured with a dial-gauge micrometer (Mitutoyo) biweekly beginning 1 week after infection. To evaluate footpad swelling, we determined the difference in measurement between the right hind footpad and the uninfected left hind footpad. Parasite burdens were counted by limiting dilution assays in which parasites were extracted from ground footpad tissue collected from individual mice. Serum was obtained 9 weeks after infection and total IgE and *Leishmania* freeze/thaw antigen-specific IgG1 and IgG2b levels were measured by ELISA. Cytokine mRNA from unstimulated popliteal lymph node cells ($1 \times 10^6$) was measured by real-time quantitative PCR.

### LCMV Infection Model

LCMV stocks were prepared and quantified as described (McCausland et al., 2007). All infections were done by i.p. injection of $1–2 \times 10^5$ PFU LCMV Armstrong per mouse. Two weeks after infection, $CD4^+$ T cells were isolated from lymph nodes and spleen with a CD4-positive isolation kit (Dynal). Staining for flow cytometry was performed with fluorophore-conjugated antibodies against B220, CD8, PD-1, CD-44, CD62L, and CD4 (Ebioscience). CXCR5 staining was performed as described in the earlier section. $CXCR5^+PD\text{-}1^{hi}CD4^+CD44^{hi}CD62L^-CD8^-B220^-$ cells were sorted with a FACS Aria (Becton Dickinson). Three-quarters of the sorted Tfh cells were fixed (as described below) for chromatin analysis and one-quarter stored in Trizol for mRNA quantification by real-time PCR. For flow cytometric analysis of germinal center B cells, lymph node and spleen cells were stained with antibodies against CD19, CD4, CD8, PNA, FAS, GL7, and IgD and analyzed on a FACS Canto (Becton Dickinson).

### Chromatin Immunoprecipitation

The detailed protocol is described in the Supplemental Information.

### Statistical Analysis

A two-tailed Student's t test was used for statistical analysis. Differences with a p value of less than 0.05 were considered significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at doi:10.1016/j.immuni.2011.12.014.

## ACKNOWLEDGMENTS

The authors thank L. Du, L. Smith, E. Yanni, Z. Yang, Y. Soo Choi, and J. Yang for expert technical assistance and M. Kubo and S. Crotty for advice and critical reading of the manuscript. This project was funded by the Burroughs Wellcome Fund (to K.M.A.), GSK National Clinician Scientist Fellowship Award and Peel Travel Fellowship Award in United Kingdom (to P.V.), NIH grants AI40127 and AI44432, and an award from the American Asthma Foundation (to A.R.). L.J.S. is a National Science Foundation Graduate Research Fellow. D.B. is the recipient of a Swiss National Science Foundation Postdoctoral Fellowship. P.V., G.S., K.M.A., and A.R. conceived the work, designed, performed and analyzed experiments, and wrote the paper; L.J.S., S.A.-W., J.I., I.M.D., and D.R.B. assisted in some of the experiments under the supervision of P.V., G.S., K.M.A., A.R., and A.H.S.; and X.H. performed some of the experiments with the model of allergic airway disease.

Received: June 1, 2011
Revised: November 18, 2011
Accepted: December 6, 2011
Published online: February 9, 2012

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