Hypersensitive site 6 of the Th2 locus control region is essential for Th2 cytokine expression

Adam Williams[a,1], Gap Ryol Lee[b,1], Charalampos G. Spilianakis[c], Soo Seok Hwang[b], Stephanie C. Eisenbarth[a,d], and Richard A. Flavell[a,e,2]

[a]Department of Immunobiology, [d]Department of Laboratory Medicine, and [e]The Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520; [b]Department of Life Science, Sogang University, Seoul 121-742, Korea; and [c]Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology, 70013 Heraklion, Crete, Greece

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The T helper type 2 (Th2) cytokine genes *Il4*, *Il5*, and *Il13* are contained within a 140-kb region of mouse chromosome 11 and their expression is controlled by a locus control region (LCR) embedded within this locus. The LCR is composed of a number of DNase I–hypersensitive sites (HSs), which are believed to encompass the regulatory core of the LCR. To determine the function of these sites, mutant mice were generated in which combinations of these HSs had been deleted from the endogenous LCR, and the effect on Th2 cytokine expression was assessed through the use of in vivo and in vitro models. These experiments revealed that, although all of the hypersensitive sites analyzed are important for appropriate LCR function, some sites are more important than others in regulating cytokine expression. Interestingly, each LCR mutation showed contrasting effects on cytokine expression, in some cases with mutants displaying opposing phenotypes between in vitro cultures and in vivo immunizations. These studies indicated that Rad50 hypersensitive site 6 was the singularly most important HS for Th2 cytokine expression, displaying consistent reductions in cytokine levels in all models tested. Furthermore analysis of chromatin modifications revealed that deletion of Rad50 hypersensitive site 6 impacted epigenetic modifications at the promoters of the *Il4*, *Il5*, and *Il13* genes as well as other regulatory sites within the Th2 locus.

Upon appropriate stimulation CD4 T cells can differentiate into numerous subsets of T helper (Th) effector cells, including Th1, Th2, and Th17, respectively (1). Th1 cells confer cell-mediated immunity against intracellular pathogens and are characterized by expression of IFN-γ and lymphotoxin-α. Th2 cells produce IL-4, IL-5, and IL-13 and assist in directing immunity against extracellular parasites and also play a role in allergic responses, including asthma (1). In the presence of antigen, IL-12 drives naive CD4 T cells to differentiate into Th1 cells, whereas stimulation in the presence of IL-4 drives Th2 differentiation. Appropriate regulation of *Il4* expression is therefore critical in the generation of effective Th2 responses.

The murine Th2 cytokine genes for *Il4*, *Il5*, and *Il13* are located within a 140-kb region on chromosome 11 flanking the DNA repair gene *Rad50* (2). In experiments using transgenic mice, a 120-kb BAC encompassing the Th2 locus was able to recapitulate appropriate expression of both IL-4 and IL-13 (but not IL-5), indicating that this BAC transgene included a locus control region (2). Locus control regions (LCRs) are defined experimentally by their ability to impart position-independent, copy number–dependent and tissue-specific expression upon linked transgenes. LCRs are cis-regulatory elements often comprising classical enhancers, insulators, boundary elements, and matrix attachment regions that are grouped into a functional unit delineated by DNase I hypersensitive sites (HSs) (3). The Th2 LCR has been mapped to a region of ∼25 kb situated within the 3′ intronic regions of the *Rad50* gene (2). Subsequently, DNase I hypersensitivity analysis of this region revealed the presence of hypersensitive sites (named Rad50 hypersensitive sites 4–7, or RHS4-7) that coincide with peaks of evolutionarily conserved DNA sequences (4, 5). In both transient transfection and transgenic experiments, these HSs functioned alone or in combination as classical enhancers (4). At the endogenous locus, RHS5 and RHS6 are present in both Th1 and Th2 cells, whereas RHS4 and RHS7 are Th2-specific (4, 5). Pretreatment of naive T cells with cyclosporin A prevents the formation of LCR RHS6-7, suggesting that they are established by an nuclear factor of activated T-cells (NFAT)-dependent pathway (5). In addition, RHS7 formation is dependent on Il4-induced STAT6 signaling (5).

The Th2 LCR is also a hotspot for epigenetic modification and during Th2 differentiation, the LCR HSs become enriched in "active" histone modifications (acetylation of histone H3 and methylation of histone H3K4), whereas, in Th1 cells the locus displays elevated levels of "repressive" histone modifications (H3-K9 methylation) (4, 5). Furthermore, whereas CpGs within RHS7 remain fully methylated in differentiated Th1 cells, they are rapidly demethylated (within 2 d) upon induction of Th2 differentiation (6).

Experiments using the chromosome conformation capture technique, which provides information about the spatial organization of chromosomal regions in vivo, have shown that T-cell receptor activation induces the formation of long-range intrachromosomal associations between the Th2 LCR and the *Il4*, *Il5*, and *Il13* promoters (7, 8). This finding suggests that the LCR coordinately regulates cytokine gene expression through direct contact with the promoter sequences (7, 8).

Currently, little is known regarding the exact function of the individual Th2 LCR HSs of the endogenous locus. Deletion of RHS7 results in decreased expression of IL-4 and IL-3, but not IL-5, from in vitro–generated Th2 cells (8). This led to the speculation that RHS7 is required for the transcriptional regulation of *Il4* and *Il13* but is less important for *Il5* expression, provided that polarizing cytokines are supplied exogenously during Th2 differentiation (8). At the DNA level, deletion of RHS7 resulted in a failure of the LCR to form normal intrachromosomal associations with the cytokine promoters in Th2 effector cells (8). Subsequently, we have shown that deletion of the entire LCR in $CD4^+$ T cells resulted in a failure to express all three of the Th2 cytokines, revealing that *Il5* is in fact under control of the LCR (9). However, analysis of these mice is confounded by the loss of the *Rad50* gene on LCR ablation (9).

Therefore, to determine the function of the remaining HS in more detail, a number of mutant mice were generated in which defined regions from the LCR were ablated while leaving the *Rad50* exonic regions intact. Analysis of mice carrying homozygous deletions revealed that each of the ablated regions was important for regulation of Th2 cytokine expression. Of the regions analyzed,

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[1]A.W. and G.R.L. contributed equally to this work.

[2]To whom corresponding should be addressed. E-mail: richard.flavell@yale.edu.

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deletion of RHS6 had the greatest impact on cytokine expression. Interestingly, the relative importance of each site was also found to be dependent upon the manner in which Th2 polarization was achieved. For example, defects seen upon in vitro polarization were much more pronounced than those seen after polarization in vivo. In addition, the RHS45 line displayed enhanced Th2 cytokine expression after in vitro polarization but displayed either equivalent or reduced cytokine levels when polarized in vivo. Together these data highlight that our understanding of signals driving CD4 effector T-cell polarization is incomplete and suggest that the multiunit Th2 LCR functions as a center to interpret diverse signals, ensuring appropriate Th2 cytokine expression in response to varied pathogenic insults.

Results

Deletion of Th2 LCR HS Does Not Affect RAD50 Expression. To analyze the function of the Th2 LCR HSs, ES cell mutants were generated by deleting regions of conserved sequences within the 3′ *Rad50* introns. These ES cells were used to generate mice constitutively lacking each region (Fig. S1). Mutant mice were backcrossed to C57BL/6J for a minimum of five times before intercrossing to generate homozygous mutants. Rad50 is an essential protein involved in DNA double-strand break repair, and although all *Rad50* exonic sequences were left intact after HS deletion, it was possible that we had disturbed *Rad50* expression (10). However, because deletion of *Rad50* is lethal and our mutant lines carrying constitutive homozygous mutations were viable and displayed no obvious defects, we believe that it is unlikely the *Rad50* expression was disturbed (11, 12). To formally prove that *Rad50* expression was unaffected, protein isolates from mutant thymocytes were subjected to Western blot analysis (Fig. S2). Probing with an anti-Rad50 antibody failed to reveal any differences in Rad50 expression between WT and mutant thymocytes.

In Vitro Polarization Reveals Defects in Cytokine Expression. To generate mutant Th2 cells under controlled conditions, we took advantage of in vitro polarization assays. Naive $CD4^+$ T cells sorted from WT and mutant mice were cultured under Th2-polarizing conditions for 5 d. Differentiated cells were restimulated overnight with plate-bound anti-CD3 antibody and cytokine levels were assayed in supernatants by ELISA (Fig. 1*A*). In separate experiments, RNA was isolated for quantification of cytokine mRNA levels (Fig. 1*B*). Upon restimulation, WT cells secreted appropriate amounts of Th2 cytokines IL-4, IL-5, and IL-13, confirming successful polarization toward the Th2 lineage (see Fig. 3*A*). As previously reported, RHS7 mutants secreted significantly lower levels of IL-4 and IL-13, but produced relatively normal levels of IL-5 (8). In contrast, both the RHS6 and RHS457 mutants were severely compromised in their ability to produce all three of the Th2 cytokines at both the protein and RNA levels. Moreover, the reduction in cytokine production in these mutants was more striking than after deletion of RHS7. Unexpectedly, the RHS45 mutant consistently showed significantly increased levels of IL-4 protein production (as well as a subtle increase in IL-5 and IL-13 expression), although this was not found to be significant at the RNA level. Because we were intrigued by the increased expression of cytokines in the RHS45 mutant line at the protein level, we performed intracellular staining to corroborate these finding using an alternative methodology. Thus, to assess cytokine expression on a per-cell basis, naive $CD4^+$ T cells were sorted from individual mice and cultured under Th2-polarizing conditions. After 5 d, cells were restimulated overnight with plate-bound anti-CD3 and then further stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence of brefeldin A. Stimulated cells were subjected to intracellular staining and analysis by flow cytometry (Fig. 2 *A* and *B*). Analysis of WT mice revealed expression of IL-4, IL-5, and IL-13, indicating that polarization toward the Th2 lineage had occurred correctly. In agreement with the ELISA data, polarized cells from RHS45 mutants consistently displayed a higher percentage of cytokine expressing cells. Interestingly, analysis of the mutant Th2 cells revealed that, although the percentage of cells expressing IL-4 and IL-13 were decreased in RHS6, RHS457, and RHS7 mutant cultures, the percentage of IL-5–expressing cells were not (Fig. 2 *A* and *B*).We hypothesize that inclusion of a different type of stimulation (PMA/ionomycin) in the intracellular staining protocol reveals a differential effect of each LCR deletion that is dependent upon the mode of stimulation; this idea is potentially supported by the in vivo data presented in the following section.

Th2 LCR Mutants Show Defects in Cytokine Expression After in Vivo Immunization with Ova/Alum. To determine the in vivo effect of the Th2 LCR HS deletions, we used a simple in vivo Th2-biased immunization model. Mice were immunized by i.p. injection of ovalbumin (Ova) adsorbed to alum; 10 d later, splenocytes were isolated and restimulated in vitro with Ova for 5 d and supernatants were analyzed by ELISA (Fig. 3*A*). In separate experiments, RNA was isolated to quantify cytokine mRNA levels (Fig. 3*B*). ELISA revealed that immunized WT mice had generated a robust Th2 immune response, as shown by expression of high levels of IL-5 and IL-13 (Fig. 3*A*). In contrast, the levels of the Th2 cytokine IL-4 were below the detection level of the assay used. As a control, we included the previously described LCR mutant line that lacks RHS7. In agreement with previously published observations, expression of IL-5 and IL-13 were significantly reduced in

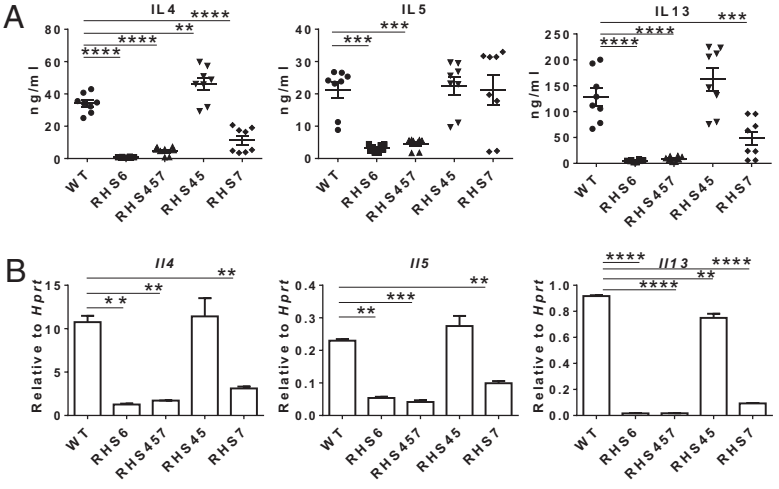

Fig. 1. Differential defects of LCR mutation on Th2 cytokine expression in vitro. (*A*) FACS-sorted naive $CD4^+$ T cells were cultured under in vitro Th2-polarizing conditions for 5 d. Live polarized cells were then restimulated for 24 h with plate-bound anti-CD3. Cytokine production was assessed by ELISA. Each point represents a single sample performed as duplicates in four independent experiments. (*B*) FACS-sorted naive $CD4^+$ T cells were cultured under in vitro Th2-polarizing conditions for 5 d and then restimulated for 24 h with plate-bound anti-CD3 before assessment of Cytokine mRNA levels by semiquantitative RT-PCR. Statistical analysis was performed by one-way ANOVA using the Dunnett's multiple comparison test. $^{****}P < 0.0001$, $^{***}P \leq 0.0009$, $^{**}P \leq 0.0043$.

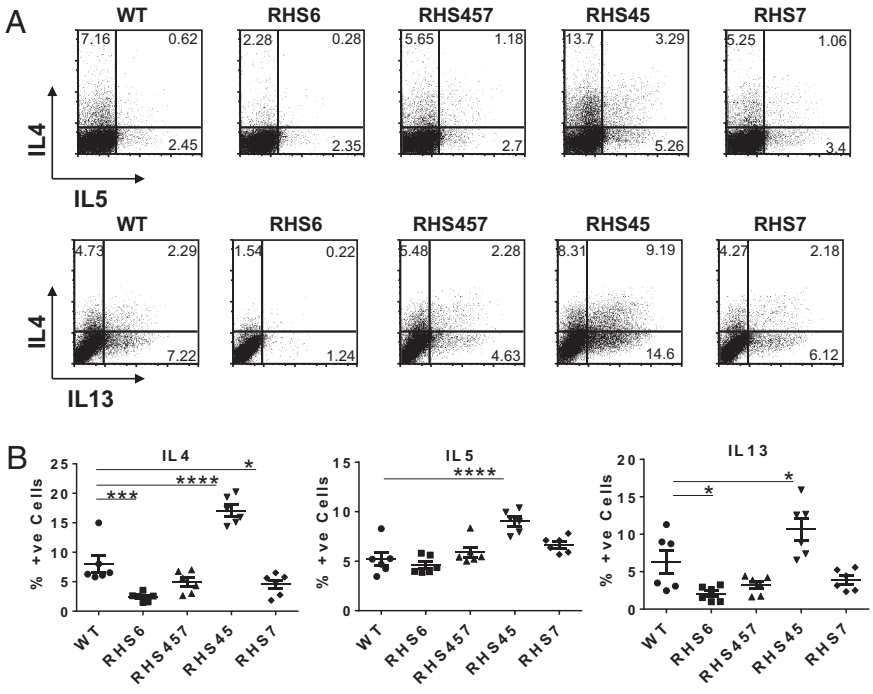

Fig. 2. RHS45-deficient mice show elevated levels of Th2 cytokine expression. (*A*) Naive CD4$^+$ T cells were sorted from individual mice and cultured under in vitro Th2-polarizing conditions for 5 d. Live polarized cells were then restimulated for 24 h on plate-bound anti-CD3 before the addition of PMA, ionomycin, and brefeldin A. After activation, cells were subjected to intracellular staining and analyzed by flow cytometry. Representative flow cytometry plots of cytokines levels as revealed by intracellular staining in each of the mutant lines. (*B*) Graphical representation of percentage of cells expressing signature cytokines as revealed by intracellular staining. Points represent cultures from individual mice in two independent experiments. Statistical analysis was performed by one-way ANOVA using the Dunnett's multiple comparison test. ****$P < 0.0001$, ***$P \leq 0.0009$, *$P \leq 0.0479$.

this line (8). Analysis of the other mutant lines revealed a similar phenotype, displaying decreased levels of both IL-5 and IL-13. Although there were no statistical differences between the different mutants, deletion of RHS6 appeared to have the greatest effect both at the protein and RNA levels, whereas deletion of RHS45 showed the least significant effect (Fig. 3 *A* and *B*). Interestingly, comparing these data with the in vitro polarized cultures reveals that the mutants behave differently in vivo and in vitro, suggesting that the relative importance of the LCR might depend upon how the T cell is activated.

Th2 LCR Mutants Show Less Severe Defects After Epicutaneous Priming. The data presented suggest the mode of T-cell stimulation determines the relative importance of the LCR. To provide further support for this hypothesis, we used a second in vivo model in which immunization was achieved through epicutaneous priming. Four days following epicutaneous priming, skin-draining (axillary) lymph nodes were removed and restimulated for 48 h with Ova-loaded antigen-presenting cells (APCs). Culture supernatants were analyzed by ELISA (Fig. 4).

WT mice displayed a robust Th2 response and secreted high levels of IL-5 and IL-13 that were comparable to those obtained after i.p. immunization with Ova/alum. The levels of IL-4 were below the sensitivity of the assay used. Surprisingly, the RHS7 and RHS45 lines produced equivalent levels of cytokines to WT mice, and the RHS457 line only displayed a mild defect in IL-13 expression. In contrast to other deletions, mice in which RHS6 had been ablated displayed a statistically significant reduction in

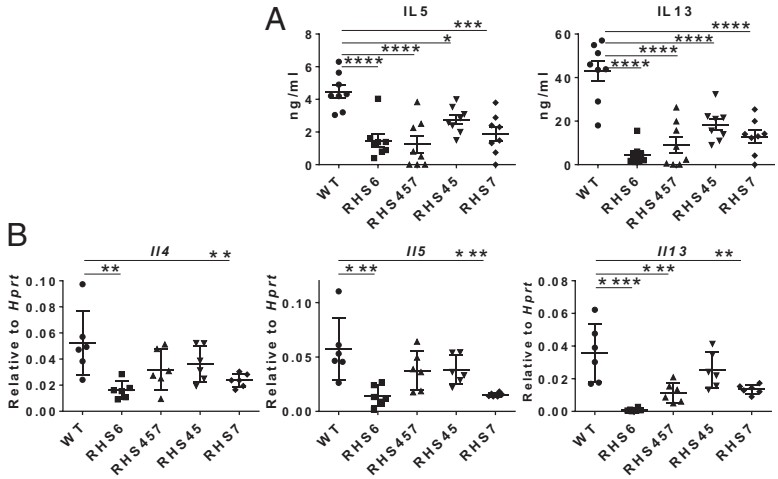

Fig. 3. Th2 LCR mutants show defective cytokine expression in vivo. (*A*) Mice were immunized with Ova adsorbed to alum administered by i.p. injection. Ten days later, splenocytes were isolated and restimulated for 5 d in the presence of Ova protein. Cytokine levels in supernatants were analyzed by ELISA. Points represent individual mice from two independent experiments. (*B*) In subsequent experiments performed as in *A*, RNA was isolated after 5 d of restimulation and cytokine mRNA levels assessed by semiquantitative RT-PCR. Statistical analysis was performed by one-way ANOVA using the Dunnett's multiple comparison test. ****$P < 0.0001$, ***$P \leq 0.0009$, **$P \leq 0.012$, *$P \leq 0.018$.

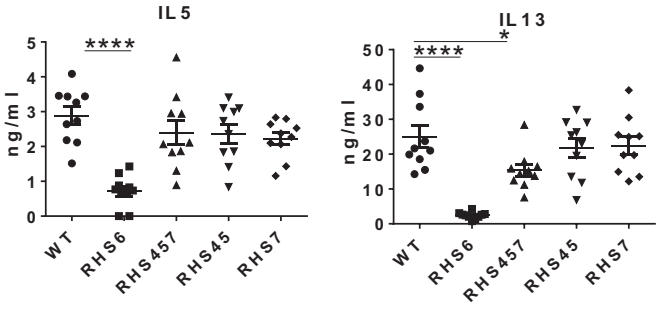

Fig. 4. Th2 LCR mutants show less severe defects after epicutaneous priming. Mice were primed epicutaneously with Ova protein. Three days after priming skin, draining lymph nodes were removed and isolated cells were restimulated for 48 h with Ova protein in the presence of splenic APCs. Cytokine levels in supernatants were analyzed by ELISA. Points represent individual mice from two independent experiments. $****P < 0.0001$, $*P \leq 0.018$.

both IL-5 and IL-13 expression. These results are strikingly different from those obtained after i.p. immunization with Ova/alum. This possibly reflects the different anatomical sites of immunization (i.e., i.p. vs. epicutaneous) or the choice of adjuvant. Alternatively, in each of the models different types of APCs or other accessory cells may be driving T-cell activation and Th2 polarization.

RSH6 Is Required for Appropriate Epigenetic Modification of the Th2 Locus in Polarized Effector Cells. The data presented clearly demonstrate that various LCR mutants respond differently depending on the model used. However, experiments in vitro and in vivo revealed that RHS6 was the singularly most important HS required for Th2 cytokine expression, regardless of the mode of polarization/stimulation. We therefore next sought to determine whether RHS6 was critical for epigenetic modification of the Th2 LCR (Fig. 5). Indeed, ChIP experiments revealed that RHS6 ablation resulted in reduced levels of active histone modifications [Pan-acetylated H3 and Histone H3 trimethyl lysine 4 (3meH3K4)] at the promoters of *Il4*, *Il5*, and *Il13* as well as known regulatory elements encompassed within the Th2 locus. These findings are similar to those previously published in total LCR deficient mice (9). In contrast, we noted that levels of the repressive histone modification, Histone H3 trimethyl lysine 27 (3meH3K27), were elevated across the Th2 locus in RHS6-deficient Th2 cells. Together these findings illustrate that RHS6 is required to establish an appropriate epigenetic landscape, normally associated with active transcription, throughout the Th2 locus and that this correlates with dramatically reduced expression of the Th2 cytokine genes.

Discussion

To dissect the role of different DNase I HSs within the Th2 LCR, mouse mutants were created in which specific regions of the LCR were ablated from the endogenous locus. Analysis of these mutants revealed the importance of each of these sites in regulating the expression of the Th2 cytokine genes in both in vitro polarization assays and in vivo immune responses. Although all sites analyzed are required for appropriate LCR function, each region appears to have unique properties that are revealed only by comparing different model systems. RHS6 mutant mice displayed the most robust defect in cytokine expression both in vitro and in vivo. Furthermore, ablation of RHS6 resulted in a switch in the epigenetic status of the Th2 locus as evidenced by a reduction in active epigenetic modifications (Pan-acetyl H3 and 3meH3K4) and an increase in "inactive" epigenetic modifications (3meH3K27). Thus, RHS6 function correlates with the establishment of an epigenetic landscape in the Th2 locus permissive for Th2 cytokine expression. However, the epigenetic change appears to be less dramatic than the transcriptional change. Interestingly, it has previously been shown that the epigenetic status of the Th2 locus does not always correlate directly with the levels of active transcription (13). Together these findings suggest that, in addition to the modulating the epigenetic status of the Th2 locus, RHS6 may directly regulate cytokine transcription, perhaps through long-range intrachromosomal associations (7, 8).

Because deletion of RHS6 resulted in the most significant reduction in cytokine expression, it is tempting to speculate that this is the most important site within the LCR. However, deletion of sequences can indirectly affect the function of remaining sites within the LCR; therefore, care must be taken when interpreting data from this type of mutation analysis. Indeed, deletion of small regions from the β-globin LCR has in some instances been shown to impact globin expression more than larger deletions encompassing the same sequence, although the reason for these discrepancies remains to be determined (14, 15).

One of the most surprising observations was the disparate results obtained from the two different in vivo models, in which the relative importance of each site for directing cytokine expression differed depending on how the immune response is initiated. For example, whereas all sites were shown to be important for i.p. Ova/alum immunization, only the RHS6 line showed significant defects in cytokine expression after epicutaneous Ova priming. One possible reason for this observation could be the differences in the immunization protocols. Lending potential support for this hypothesis is the observation that i.p. Ova/alum immunization is dependent upon IL-4, whereas epicutaneous priming is independent of IL-4 but is instead dependent upon IL-13 (16, 17). How this difference relates to the function of individual LCR elements is currently unknown. Perhaps in the different models APCs provide quantitatively or qualitatively different signals to the T cells, which in turn operate through different regions of the LCR to drive cytokine gene expression. With this possibility in mind, the in vitro results become particularly interesting. Here the differences between the various mutants are much more pronounced. For example, whereas RHS7 is required for optimal IL-4 and IL-13 expression but is partly dispensable for IL-5 protein expression, deletion of RHS6 and RHS457 results in almost complete loss of expression of all three cytokines. At the opposite end of the spectrum, the RHS45 line displayed increased levels of cytokine expression in vitro. In transient transfection assays, RHS5 has previously been shown to decrease transcriptional output from a minimal *Il4* promoter, leading to the possibility that RHS5 may possess a transcriptional silencing capacity (4). However, no such enhancement was observed in RHS45 mice in vivo, again highlighting the differences between the various models used.

The divergent phenotypes noted between in vitro and in vivo models highlight that our current understanding of how effector T cells acquire and realize a Th2 phenotype is still incomplete. These findings are not unprecedented; indeed, Th2 differentiation itself is known to display different requirements in vitro and in vivo. For example, whereas cells lacking IL-4 or STAT6 fail to differentiate into Th2 cells in vitro, they are able to undergo robust Th2 differentiation in vivo. Moreover, to add to this complexity, Th2 differentiation in vivo can be driven by multiple pathways. For example, although Th2 responses to *Nippostrongylus brasiliensis* can occur independently of IL-4 or STAT6, Th2 responses to *Trichuris muris* are entirely dependent upon IL-4 (18, 19). In addition, as previously mentioned, i.p. immunization with Ova/alum and epicutaneous priming with Ova showed different requirements for IL-4 and IL-13 in vivo, indicating that polarization in response to priming with a simple antigen can also occur via different mechanisms (16, 17). Therefore, just as there are multiple mechanisms to direct Th2 differentiation, it is possible that there are multiple pathways to drive Th2 cytokine expression. We propose that although the Th2 LCR

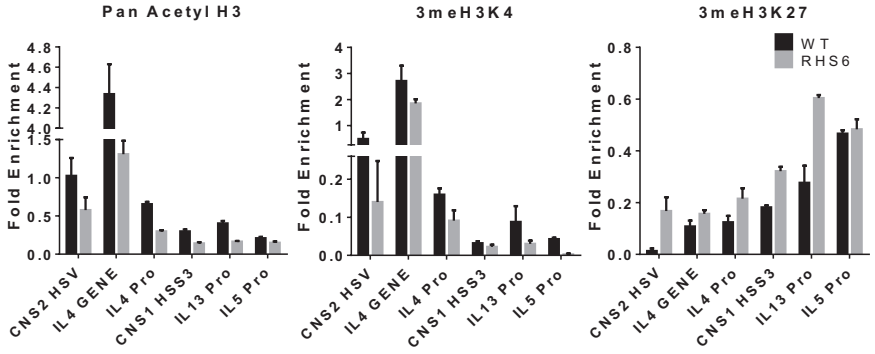

Fig. 5. RHS6 mutant mice show epigenetic alterations along the Th2 locus. ChIP analysis of the Th2 locus in WT and RHS6 mutant cells. Sorted naive $CD4^+$ T cells were polarized under Th2 conditions for 5 d and then restimulated overnight with anti-CD3 before being fixed in formaldehyde and then subjected to ChIP analysis. Graphs show levels of, pan-acetyl H3, 3meH3K4, and 3meH3K27 along the Th2 locus gene as determined by real-time PCR. Data represent average fold enrichment values of two independent immunoprecipitations and error bars show SEM. See *Materials and Methods* for calculations used to determine fold enrichment values.

is a single functional unit, each HS plays a unique role, imparting the LCR with the flexibility to interpret a range of signals initiated in responses to varying immunologic insults. With respect to proteins important for LCR function, it has been shown that LCR hypersensitivity is NFAT-dependent (5). Furthermore, RHS7 formation and demethylation is dependent upon STAT6 signaling. ChIP analysis has also revealed that GATA binding protein 3 (GATA3) binds to RHS4, RHS5, and RHS6 in Th2 cells (1, 5). Surprisingly, in contrast to transgenic experiments using an Il4 minilocus, expression of a transgene linked to the LCR was not enhanced by enforced GATA3 expression (2, 20). However, at the endogenous locus, GATA3 is required for the formation of long-range intrachromosomal interactions involving the LCR, indicating that GATA3 may function as more than a simple transcriptional activator (7). Thus, our understanding of factors known to bind to the LCR is obviously lacking and currently cannot account for the flexibility of LCR function.

In conclusion, determining how each LCR HS contributes to cytokine regulation in different models and identification of transcription factors important in this process may provide a more complete understanding of $CD4^+$ T-cell differentiation and how Th2 cytokine gene expression is tightly regulated in response to various pathogenic insults.

Materials and Methods

Generation of RHS45, RHS457, and RHS6 KO Mice. BAC DNA containing the *rad50* gene (B172) was used for construction of the targeting vectors (2). The generation of RHS7 KO mice was previously described (8). To generate the RHS45 line, a targeting vector was designed to replace two regions of RHS4 and RHS5 without disrupting exons 20 and 21 of Rad50. To this end, a 6.7-kb HindIII-NcoI fragment (containing exons 16–19 of mouse *rad50* gene) and a 2.0-kb fragment containing exons 20 and 21 were subcloned as the 5′ arm and a 3.0-kb fragment containing exons 22 and 23 was subcloned as the 3′ arm into pEasyFlox (a gift from Dr. K. Rajewsky, The Max Delbrück Center for Molecular Medicine, Berlin). This replaced the two regions of RHS4 and RHS5 (2.4 kb, 34751–37196 bp, and 7.4 kb, 39132–46531-bp fragments from translation start site of *rad50* gene) with the neomycin-resistance cassette flanked by two Cre recombinase *loxP* sites. A thymidine kinase gene was used for negative selection of clones with random integration of the targeting vector. To generate the RHS457 line, the RHS45 construct was used to retarget ES cells in which RHS7 had already been deleted. This allowed simultaneous deletion of RHS4, 5, and 7 without disrupting exonic sequences of the Rad50 gene. To generate the RHS6 line, a vector was designed to delete two regions of RHS6 without disrupting exon 22 of the RAD50 gene. To this end, a 3.1-kb fragment from intron 21 of mouse *rad50* gene was subcloned as the 5′ arm, and a 1.2-kb fragment containing exon 22 and a 4.8-kb fragment (containing exons 23–25) were subcloned as the 3′ arm into pEasyFlox. This vector replaces both regions of RHS6 (3.9 kb, 46438–50371 bp, and 0.3 kb, 51594–51935-bp fragments from translation start site of *rad50* gene) with the neomycin-resistance cassette flanked by two *loxP* sites. A thymidine kinase gene was used for negative selection of clones with random integration of the targeting vector. Each vector was linearized by digestion with NotI and introduced into the TC1 embryonic stem cell line (a gift from P. Leder, Harvard University, Cambridge, MA) by electroporation. Clones resistant to G418 and gancyclovir were selected, and homologous recombination was confirmed by Southern blot analysis. In the cases of RHS7, RHS45, and RHS457, neomycin was deleted in vitro by transfecting the Cre expression vector phosphoglycerate kinase-Cre (a gift from Dr. K. Rajewsky) into targeted embryonic stem cells. Clones with the correct deletion were screened by Southern blot analysis. For each line, two independently targeted ES clones were injected into C57BL/6 blastocysts and transferred into pseudopregnant foster mothers; the resulting male chimeric mice were bred to C57BL/6 females to obtain heterozygous mice. Germ-line transmission was verified for both embryonic stem clones by Southern blot analysis of DNA obtained from tail tissue from F1 offspring. Heterozygous RHS6 mice were bred to transgenic mice ubiquitously expressing Cre recombinase (generated in our facility) to allow germ-line deletion of the neomycin resistance gene. Each line was backcrossed a minimum of five times to C57BL/6 before intercrossing to generate homozygous mice. All protocols used in this study were approved by the Yale Institutional Animal Care and Use Committee.

Western Blot. Electrophoresis of proteins was performed with the NuPAGE system (Invitrogen) in accordance with the manufacturer's protocol. In brief, thymocytes were resuspended in lysis buffer (Cell Signaling) containing a protease inhibitor mixture (Roche). Lysates from 8×10^6 thymocytes were separated on a NuPAGE gel and transferred to a PVDF membrane by electroblotting. To detect Rad50, a rabbit polyclonal anti-Rad50 antibody was used (Abcam). A goat polyclonal anti-actin antibody (Santa Cruz) was used as a loading control.

Ova Alum Immunization. For i.p. immunization, 6- to 8-wk-old mice were injected intraperitoneally on day 0 with 50 μg of ovalbumin (Grade V; Sigma) adsorbed on 4 mg of Imject alum. After 10 d, splenocytes were harvested, red blood cells were lysed with ammonium chloride and potassium solution, and 1×10^7 splenocytes were stimulated in the presence or absence of 200 μg/mL of Ova.

Epicutaneous Priming. On day zero, the backs of mice were shaved with electric clippers. On day one, Ova (100 μg) was applied to a Band-Aid and adhered to the shaven area using Mastisol liquid adhesive (Ferndale Laboratories, Inc). On day four, draining lymph nodes (brachial/axillary) were removed and single-cell suspensions were generated. A total of 5×10^6 lymph node cells were cultured with 5×10^6 APCs (*Generation of APCs*) in 1 mL in the presence or absence of 200 μg/mL of Ova for 48 h.

Generation of APCs. Syngenic T-cell–depleted splenocytes were used as APCs and were prepared by complement lysis using antibodies to CD4 (GK1.5), CD8 (TIB 105), and Thy1 (Y19), followed by treatment with mitomycin C.

Cell Sorting and in Vitro Th2 Polarization Cultures. Spleen and lymph nodes were collected from 6- to 8-wk-old mice, single-cell suspensions were

generated, and CD4 T-cell enrichment was performed with CD4-MACS beads and LS columns according to the manufacturers' instructions. After enrichment cells were stained with anti-CD4 APC (Biolegend), anti-CD25 phycoerythrin (PE) (Biolegend), anti-NK1.1 PE (BD Biosciences), anti-CD44 PE-CY5 (BD Biosciences), and anti-CD62L FITC (Biolegend). Naive T cells (CD4+/CD25-/NK1.1-/CD62L High/CD44 Low) were sorted using a FACSVantage. For Th2 polarizations, 1.5×10^6 sorted naive CD4 T cells were cultured on plates precoated with anti-CD3 (10 μg/mL) and anti-CD28 (2 μg/mL) in 2 mL of Clicks media supplemented with 10% (vol/vol) FBS, Il4 (20 ng/mL), IL-2 (0.1 ng/mL), and anti-IFN-γ (10 μg/mL). After 5 d of culture, live cells were isolated using an LSM gradient (MP Biomedicals), and 2×10^6 polarized cells were cultured in 1 mL on anti-CD3 (10 μg/mL) coated plates for 24 h before the supernatants were removed for ELISA.

ELISA. Antibody pairs were purchased from Pharmingen (Il4 and Il5) or eBioscience (Il13). ELISAs were performed as recommended by the manufacturers.

RNA Extraction and QPCR. RNA from cells was extracted using TRIzol (Invitrogen) and subjected to reverse transcription with SuperScript II (Invitrogen) using an oligo(dT) primer in accordance with the manufacturer's protocol. Finally, cDNA was quantified using commercially available primer/probe sets (Applied Biosystems) by real-time PCR and analyzed with the ΔC_t (change in cycle threshold) method. Results were normalized to control genes quantified in parallel amplification reactions during each PCR quantification. Results are presented as levels relative to hypoxanthine phosphoribosyltransferase 1 (*Hprt*).

Intracellular Staining and Flow Cytometry. Polarized cells were prepared and restimulated as discussed previously. After restimulation, PMA (10 ng/mL), ionomycin (1 μM), and brefeldin A (10 μg/mL) were added to the medium. After 5 h, the cells were harvested and stained with anti-CD45.2 (Biolegend). The cells were washed with 1× PBS and fixed on ice with 4% (wt/vol) paraformaldehyde (PFA) in PBS for 20 min, followed by permeabilization with 0.1% saponin. Intracellular staining was performed using anti-Il4 (BD Biosciences), anti-Il5 (BD Biosciences), and anti-Il13 (eBioscience) antibodies before acquisition using a FACSCalibur flow cytometer.

ChIP. Cultured or sorted populations were resuspended in warm clicks media at a concentration of 1×10^6 cells/mL. Cells were fixed with 1% formaldehyde for 10 min at 37 °C. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were washed twice with ice-cold PBS. Samples were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris·HCl pH 8.0) containing protease inhibitors (Roche) at a concentration of 1×10^7 cells/mL and sonicated using a Bioruptor (Diagenode). Sonicated samples were checked on 1% agarose gel and a 50-μL sample was reserved as input. Samples were split into aliquots containing 1×10^6 cells, diluted to 1×10^6 cells/mL in dilution buffer [0.1% SDS, 1.1% (wt/vol) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris·HCl pH 8.0, 167 mM NaCl] and precleared with protein A/salmon sperm or protein G/salmon sperm agarose beads (Upstate). Samples were incubated overnight with antibodies at 4 °C with rotation. Nonspecific rabbit IgG was included as a control antibody. Antibody–chromatin complexes were captured with protein A/salmon sperm or protein G/salmon sperm agarose beads for 4 h at 4 °C with rotation. Beads were washed once with each of the following buffers: low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl pH 8.0, 150 mM NaCl), high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris·HCl pH 8.0, 500 mM NaCl), LiCl (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris·HCl pH 8.0, 150 mM NaCl), and twice with 1 mM EDTA, 10 mM Tris·HCl pH 8.0. DNA–protein complexes were eluted with 1% SDS, 100 mM $NaHCO_3$, and cross-linking reversed by incubating samples at 65 °C overnight in the presence of 0.2 M NaCl. Samples were treated with RNase (20 μg/mL) and then with proteinase K (40 μg/mL) for 1 h. DNA was phenol-extracted and ethanol-precipitated in the presence glycogen (20 ng/mL). Samples were analyzed by real-time PCR (*ChIP Real-Time PCR*). The following antibodies were used for immunoprecipitation: anti-Histone-H3-trimethyl-K4 (Abcam ab8580), (Abcam ab9050), and anti-acetyl-Histone-H3 (Upstate 06-599), anti-Histone-H3-tri methyl-K27 (Abcam 6002), and normal rabbit IgG (Upstate 12-370).

ChIP Real-Time PCR. DNA from ChIP samples was diluted 1/10 except for input DNA, which was diluted 1/25. Of this dilution, 6 μL was combined with KAPA SYBR FAST Universal 2 × master mix (KAPA Biosystems), and 5 pmol of each primer in a total volume of 20 μL. PCR reactions were run on a 7900HT fast real-time PCR system (Applied Bioscience). Using CT values obtained from real-time PCR, the following equation was used to determine the relative enrichment of each modification: $[1/2^{(\text{I.P.ct} - \text{Input.ct})}] - [1/2^{(\text{IgG.ct} - \text{Input.ct})}]$. Values were then normalized to the control gene glucose-6-phosphate dehydrogenase (G6PD). The following primers (named according to closest genomic "landmark") were used for real-time PCR analysis of chromatin templates. The final three primer sets listed were previously published (13).

Il4 promoter: TCCCTTGGTTTCAGCAACTT/GACAACTAGCTGGGGGTTGA

Il5 promoter: GGCCTTCAGCAAAGGAAGAG/ACCCTGAGTTTCAGGACTCG

Il13 promoter: GAATTACTGGGGCGGAAGTT/GGCCGCTAAAGGAAAGAGTC

G6PD: CTAGTTTGGCTTCGGAGCTG/CCGCACTAAAACACCCAAGT

CNS2 HSV: ATCAGAACATCACGTCGTCT/ATGCCAACCTGAAGAACTAAC

IL-4 gene body: GGGTGTGAATAAGCCATATTG/CCCAGCGTTTACATGAGC

CNS1 HS3: AGGGTCTCCAGAAGATTAGCC/TCAAGCCTGATGACCTATGC

Statistical Analysis. Where presented statistical analysis was performed using GraphPad Prism 6 by one-way ANOVA using the Dunnett's multiple comparison test.

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