

Mast cell IL-4 expression is regulated by Ikaros and influences encephalitogenic Th1 responses in EAE

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When exposed to a pathogen, a naive CD4⁺ T cell is forced to make a cell fate decision that leads to a polarized population of Th1 IFN- γ - or Th2 IL-4- producing cells. Although IL-4 has traditionally been considered a factor that promotes Th2 cell differentiation, recent evidence has demonstrated that the site and timing of IL-4 expression in an immune response determines its ultimate effects on CD4⁺ T cell fate. Using a mast cell (MC) reconstitution model, we demonstrate that MC-derived IL-4 promoted Th1 responses in vivo. Furthermore, MCs from genetically disparate mouse strains varied in their potential for IL-4 expression. Independent of the activation mode, MCs from Th1-prone C57BL/6 mice exhibited a more robust *Il4* response than did the Th2-prone strain Balb/c. The hierarchy of IL-4 expression potential was directly associated with the degree of basal chromatin accessibility at *cis*-regulatory elements conserved noncoding sequence-1 and V_A enhancer within the Th2 locus. GATA1/2 and Ikaros, factors with opposing roles in chromatin remodeling, acted at these sites. We propose that GATA and Ikaros proteins coordinately fine-tune accessibility at the *Il4* locus during development to variably regulate IL-4 expression. These events likely contribute to the genetically determined heterogeneity in Th1 responses that underlie susceptibility to many diseases.

Introduction

IL-4 is a multifunctional cytokine that regulates both innate and adaptive immunity (1). Cells of several lineages produce IL-4, including CD4 and CD8 T cells, NKT cells, eosinophils, mast cells (MCs), and basophils. It is likely that expression by each cell type contributes to distinct as well as overlapping physiologic responses. IL-4 is perhaps best known for its role in promoting Th2 cell differentiation and limiting Th1 responses; however, recent evidence shows that IL-4 can also direct Th1 responses (2). IL-4 can act on a wide variety of other target cells including B cells, macrophages, DCs, and MCs as well as nonimmune cells such as epithelial cells and goblet cells (3–6). This broad range of effects necessitates strict controls on the expression of this cytokine. Inappropriate expression is associated with allergic disease, autoimmunity, and failure to clear certain infections (7).

Most information regarding mechanisms of IL-4 gene (*Il4*) regulation comes from studies in Th cells and MCs. In addition to acute signals that elicit active *Il4* transcription in both cell types, a crucial determinant of IL-4 expression is locus accessibility acquired during development (8). *Il4* is closely linked to the genes encoding IL-5 (*Il5*) and IL-13 (*Il13*). These genes are clustered within an approximately 200-kb region, termed the Th2 locus, on chromosome 11 in mice and chromosome 5 in humans. During Th cell differentiation, the Th2 locus undergoes a series of chromatin modifications that confer the relative potential for transcription

from *Il4* (8). DNase I hypersensitive site (HS) analyses and cross-species sequence comparisons have allowed the identification of a number of *cis*-regulatory elements implicated in controlling *Il4* expression. Included among these are regions between *Il13* and *Il4* (conserved noncoding sequence-1 [CNS-1]), within the *Il4* promoter (HS I) and second intron (intronic enhancer [IE]), and distal to *Il4* (HS IV, V, and V_A) (9–13). In addition, a locus control region is located upstream of *Il13* at the 3' end of *Rad50* (14). The histones associated with these sites become hyperacetylated in developing Th2 cells, correlating with the potential to express IL-4, whereas the Th2 locus is hypoacetylated and relegated to pericentromeric heterochromatin in Th1 cells (15–17). Resting bone marrow-derived MCs (BMMCs) express most of the same HS sites as Th2 cells within the *Il4* locus. Although most currently defined sites have enhancing activity, Rao and colleagues have defined HS IV as a conserved silencer of *Il4* in both T cells and MCs (9).

The *Il4* regulation paradigm is based primarily on results of experiments utilizing T cells and MCs from only 2 common strains of mice: C57BL/6 (B6) and Balb/c. These strains have been useful in studying genetically based disease susceptibility due to their disparate immune responses to some antigens, events perhaps most clearly manifested in *Leishmania major* infection (18). B6 mice clear the infection due to a Th1-dominated response, whereas Balb/c mice fail to clear the parasite despite a strong but nonprotective Th2-skewed response. Most studies have focused on possible intrinsic differences in the response of B6- versus Balb/c-derived T cells, such as instability in expression of the IL-12 receptor β 2 subunit in Balb/c T cells (19). We considered the possibility that strain-specific differences in bystander cell cytokine expression exist that modulate the Th1/Th2 balance. A genetically determined difference in the kinetics or level of IL-4 expression by MCs, for example, could directly alter the character of the T cell response or act indirectly through effects on DC

Nonstandard abbreviations used: B6, C57BL/6; BMMC, bone marrow-derived MC; CD40L, CD40 ligand; ChIP, chromatin immunoprecipitation; CNS-1, conserved noncoding sequence-1; Fc ϵ RI, high affinity IgE receptor; HDAC, histone deacetylase; HS, hypersensitive site; IE, intronic enhancer; MC, mast cell; MOG, myelin oligodendrocyte glycoprotein peptide; NuRD, nucleosome remodeler and deacetylase; V_AE, V_A enhancer; W/W^v, WBB6-F1/J-Kit^W/Kit^{Wv}.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 116:1327–1336 (2006). doi:10.1172/JCI27227.

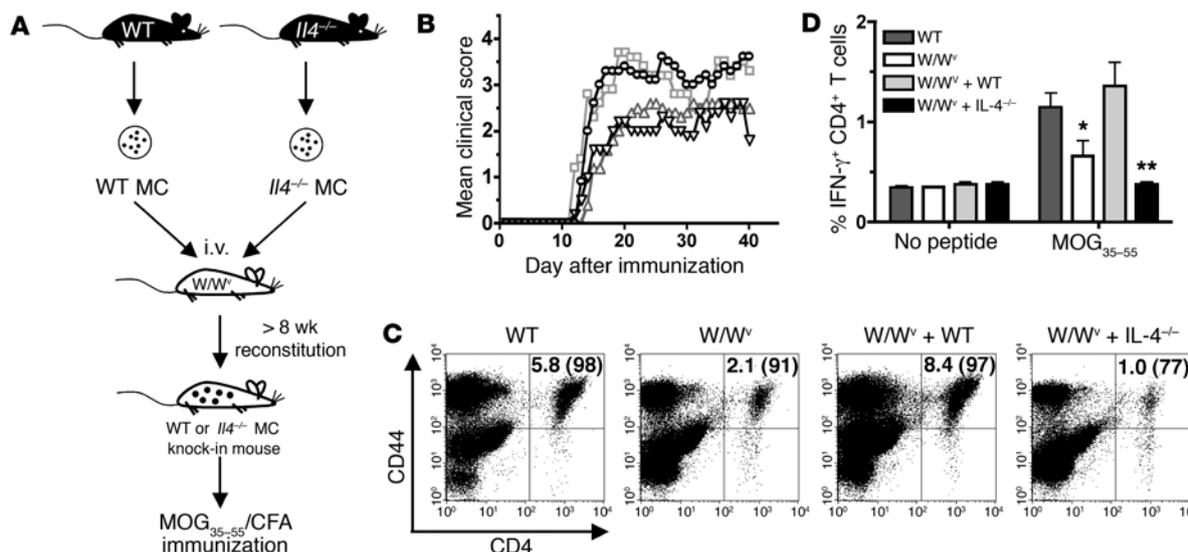


Figure 1 MC-derived IL-4 enhances the severity of EAE by promoting the generation of MOG₃₅₋₅₅-specific IFN-γ-expressing Th1 cells. **(A)** Diagram of the MC reconstitution protocol. MCs were generated by in vitro differentiation and transferred i.v. to MC-deficient W/W^v mice. **(B)** Mice were immunized with MOG₃₅₋₅₅ peptide to induce EAE and monitored daily for clinical signs of paralysis. Mean clinical scores were determined daily (*n* = 5 per group). Squares, WT littermate controls; triangles, W/W^v; inverted triangles, W/W^v + IL-4^{-/-} MC; circles, W/W^v + CD40L^{-/-} MC. W/W^v and W/W^v + IL-4^{-/-} MC mice exhibited significantly reduced EAE severity compared with WT littermate controls (*P* < 0.01). **(C)** WT, W/W^v, and W/W^v mice reconstituted with B6 or IL-4^{-/-} BMMCs were perfused at 11 days after immunization, and flow cytometric analysis of CNS-infiltrating CD44^{hi} CD4 T cells was performed. Numbers in quadrants represent the percentage of CD4 T cells; numbers in parenthesis indicate the percentage of CD4 T cells that are CD44^{hi}. **(D)** Antigen-specific CD4⁺ T cell responses were measured by intracellular cytokine staining for IFN-γ after a brief in vitro restimulation of splenocytes with MOG₃₅₋₅₅ peptide. **P* < 0.05 versus WT; ***P* < 0.01 versus W/W^v + WT. For reconstitution in **C** and **D**, B6-derived BMMCs were used as controls for IL-4^{-/-} BMMCs, whereas WBB6-F1/J-Kit^W/Kit^W WT littermates were used as controls for W/W^v mice.

maturation. In this report, we demonstrate that MC IL-4 was critical to the generation of myelin-specific Th1 cells and contributed to severe EAE, a murine model of MS. Furthermore, we show that there were strain-specific differences in murine MC *Il4* expression potential that may influence the heterogeneity of Th1 versus Th2 responses. Variability in the expression of *Il4* occurred irrespective of the mode of activation and correlated with distinct patterns of basal H3 and H4 histone acetylation at several sites within the Th2 locus, including CNS-1 and V_A enhancer (V_AE) elements. GATA1, GATA2, and Ikaros, DNA-binding proteins with established roles in chromatin remodeling, associated with CNS-1 and V_AE in vivo. We provide direct evidence that Ikaros has a suppressive role in MC *Il4* transcription, as Ikaros^{null} MCs had elevated IL-4 mRNA expression and chromatin accessibility across the Th2 locus. Our data is consistent with a model whereby the interplay of GATA and Ikaros proteins determines the variable potential for MC *Il4* expression, which subsequently exerts a direct effect on Th1/Th2 responses.

Results

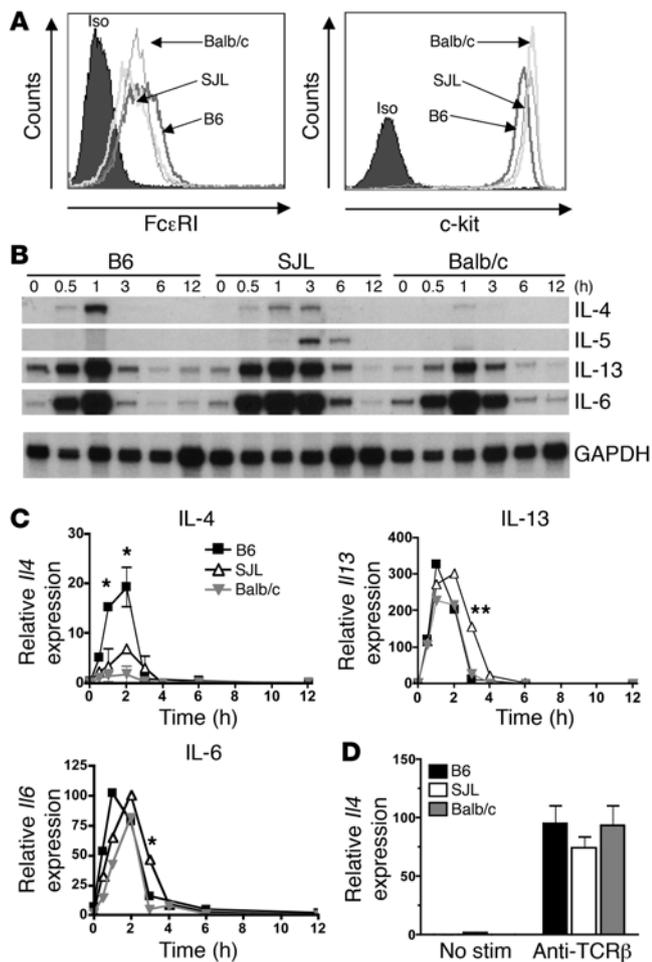
MC-derived IL-4 influences EAE severity by enhancing encephalitogenicity of Th1 cells. We hypothesized that MCs, by virtue of their ability to express cytokines that modulate T cell responses, have a role in adaptive immune responses (20). Consistent with this idea are results of previous studies showing that MC-derived TNF-α regulates in vitro T cell reactivity (21) and that MCs are essential for optimal generation of encephalitogenic Th1 cells during EAE (22). Although IL-4 is best known as a Th2-polarizing cytokine, IL-4 can also have distinct effects on Th1 responses (2, 23, 24). Thus it

was of interest to investigate whether MC IL-4 expression could modulate the quality of a Th1-mediated response.

Using a MC knock-in model in which MC-deficient WBB6-F1/J-Kit^W-Kit^W (W/W^v) mice were selectively reconstituted with either WT or IL-4^{-/-} BMMCs (referred to as W/W^v + WT and W/W^v + IL-4^{-/-}, respectively; Figure 1A), we assessed the specific effects of MC IL-4 on myelin oligodendrocyte glycoprotein peptide-induced (MOG₃₅₋₅₅-induced) EAE. As previously reported, W/W^v mice exhibited a significantly reduced overall mean clinical score compared with WT littermate controls (*P* < 0.01; Figure 1B). Reconstitution of W/W^v mice with MCs deficient in CD40 ligand (CD40L), a cell surface costimulatory molecule expressed on several cell types, restores disease to WT levels and served as a positive control in this experiment (25). In contrast, mice reconstituted with IL-4^{-/-} MCs failed to display severe disease.

Development of EAE and MS is dependent upon trafficking of activated, myelin-specific T cells to the CNS. Consistent with reduced disease severity, reduced CNS infiltration of CD4 T cells was observed in W/W^v and W/W^v + IL-4^{-/-} mice compared with WT and W/W^v + WT mice (Figure 1C). Furthermore, appreciably fewer T cells entering the CNS of W/W^v and W/W^v + IL-4^{-/-} mice were activated, as measured by high CD44 surface expression. Together, these results suggest that MC IL-4 promotes encephalitogenic Th1 responses.

MOG₃₅₋₅₅-specific T cell responses were also evaluated in WT and reconstituted mice. As shown in Figure 1D, W/W^v T cell responses were reduced relative to WT as previously reported (22). While WT BMMCs restored the frequency of MOG₃₅₋₅₅-specific IFN-γ-producing CD4 T cells, IL-4^{-/-} BMMCs did not (*P* < 0.01).

**Figure 2**

MCs exhibit strain-specific differences in *Il4* expression after Fc ϵ RI cross-linking. (A) Surface expression of Fc ϵ RI and c-kit on BMMCs from B6, Balb/c, and SJL mice after 6–8 weeks of culture in IL-3 and SCF. The mean fluorescent intensity of both c-kit and Fc ϵ RI is essentially equivalent in all strains. Iso, isotype. (B and C) Kinetics of BMMC cytokine mRNA expression after Fc ϵ RI cross-linking. Ribonuclease protection assays were performed to measure cytokine transcripts from B6, Balb/c, and SJL MCs at resting state (0 hours) and at various times after stimulation by Fc ϵ RI cross-linking. Ribonuclease protection assays were visualized by autoradiography after 16 hours (*Il5*, *Il6*, *Il13*, and *Gapdh*) or 5 days (*Il4*) and quantified by phosphorimaging ($n = 2–4$ per time point). mRNA levels were expressed relative to the housekeeping gene GAPDH and calculated as (cytokine intensity/GAPDH intensity) $\times 100$. * $P < 0.05$; ** $P < 0.01$ (ANOVA followed by Bonferroni's multiple comparison test). (D) Freshly isolated naive CD4 $^{+}$ T cells from B6, Balb/c, and SJL mice were cultured under Th2 skewing conditions for 7 days and restimulated with plate-bound anti-TCR β . *Il4* expression was measured and quantified as in C. Two independent experiments were performed. stim, stimulation.

tions between strains. For example, appreciable *Il5* expression was observed only in SJL MCs.

To examine whether this difference was also evident in T cells, naive CD4 $^{+}$ T cells from the same strains were isolated, differentiated under Th2 skewing conditions, and analyzed for cytokine expression. Unlike BMMCs, Th2 cells cultured in vitro showed no significant strain-specific variation in expression of IL-4 mRNA after restimulation through the T cell receptor (Figure 2D).

B6-derived BMMCs express higher levels of IL-4 mRNA irrespective of activating agonist. The unique profiles of MC *Il4* expression could be intrinsic to Fc ϵ RI signaling; alternatively, they may reflect more global differences in basal chromatin accessibility at the *Il4* locus, leading to distinct expression potentials regardless of the mode of stimulation. To distinguish between these possibilities, the expression of IL-4 mRNA was compared after TLR-induced signaling. TLR2 and TLR4 mRNA expression was confirmed in cells derived from all 3 strains (Figure 3A). Cells were then stimulated with peptidoglycan (a TLR2 agonist), LPS (a TLR4 agonist), or heat-killed *Mycobacterium tuberculosis*, which can signal through both TLR2 and TLR4. B6-derived MCs expressed higher levels of IL-4 mRNA in response to all agonists compared with Balb/c MCs (Figure 3, B and C). This result was consistent with the idea that B6 MCs (IL-4 hi) have a greater potential for IL-4 expression irrespective of the activating stimulus.

Il4 expression potential correlates with Th2 locus accessibility. Guo et al. demonstrated that the probability of a Th2 cell producing relatively high or low levels of IL-4 directly correlates with the degree of histone acetylation, a marker of chromatin accessibility, at the Th2 cytokine locus (27). To examine whether strain-based differences in the ability of MCs to express IL-4 are reflected in distinct chromatin states that affect accessibility within the *Il4* locus, histone acetylation was evaluated in resting MCs by chromatin immunoprecipitation (ChIP) using antibodies specific for acetylated histones H3 and H4. IP templates were analyzed by PCR using primers specific for previously defined regulatory elements within the Th2 locus (Figure 4A). Several differences were noted in histone H3 and H4 acetylation between strains (Figure 4, B–D). Of particular interest is the consistent and striking difference in acetylation observed at CNS-1 and V $_A$ E in the IL-4 hi B6 and IL-4 int SJL strains relative to Balb/c. Both of these elements are necessary for

Similar results were obtained in experiments using immunization with OVA $_{323–339}$, a nonencephalitogenic peptide (data not shown).

MCs, but not Th2 cells, exhibit strain-specific differences in IL4 expression. The demonstration that MC IL-4 enhanced Th1 responses raised the possibility that the propensity for Th heterogeneity in various strains of mice might be due to intrinsic differences in the magnitude of MC IL-4 expression. For example, MCs from Th1-prone mice may have relatively higher IL-4 expression compared with a Th2-dominated strain. MCs from the B6 and Balb/c strains were chosen to examine potential strain-specific differences in MC *Il4* expression due to their dichotomous responses to *L. major* infection (18). The SJL strain was also examined as it has documented defects in T cell IL-4 expression and IgE production in vivo (26). MCs were differentiated from bone marrow by culturing in the presence of SCF and IL-3. BMMCs from all 3 strains expressed comparable levels of c-kit and the high affinity IgE receptor (Fc ϵ RI) after 6–8 weeks of culture (Figure 2A). Cells were activated by cross-linking of Fc ϵ RI, and IL-4 mRNA levels were measured at various times after stimulation. As shown in Figure 2, B and C, B6 BMMCs exhibited quick and relatively robust expression of *Il4* compared with BMMCs from Balb/c or SJL mice ($P < 0.05$ at 1 and 2 hours). SJL BMMCs showed intermediate and more prolonged expression of *Il4*, whereas Balb/c MCs displayed a relatively weak expression profile, demonstrating the strain-specific variations in the duration and magnitude of the IL-4 response. The expression of other genes such as *Il5*, *Il13*, and *Il6* also displayed notable varia-

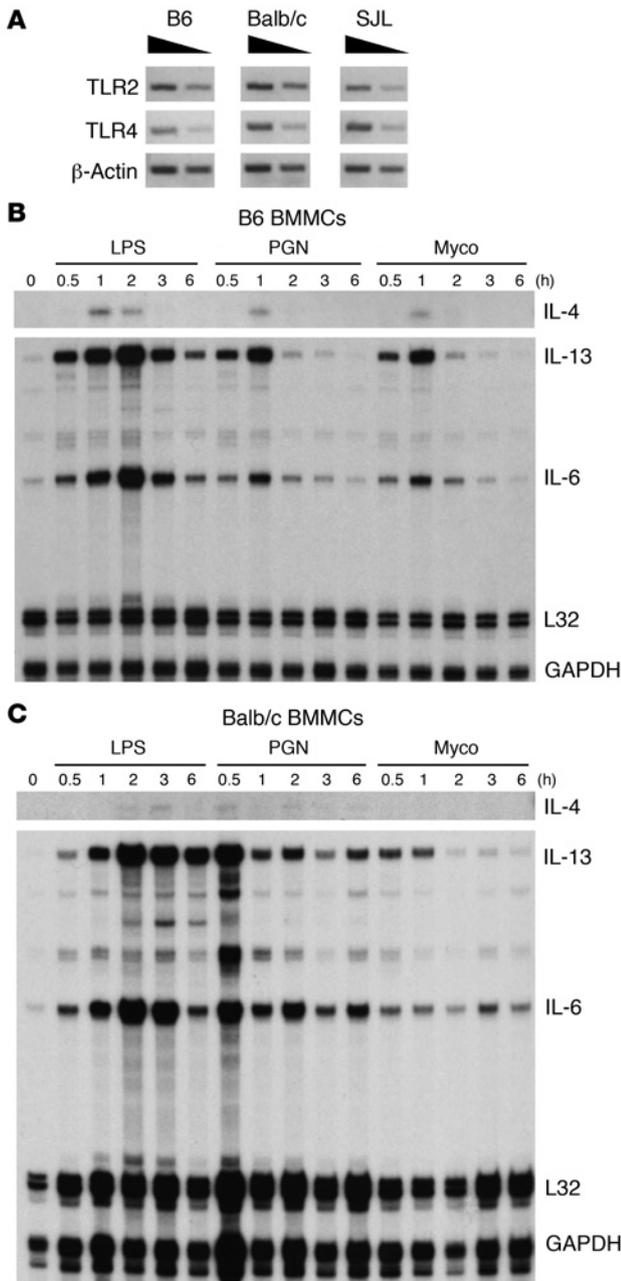


Figure 3

Strain-specific differences in B6 versus Balb/c *Il4* expression are evident after TLR stimulation. (A) TLR2 and TLR4 expression was confirmed in resting BMMCs by RT-PCR. (B and C) B6 (B) and Balb/c BMMCs (C) were stimulated with LPS, peptidoglycan (PGN), or *M. tuberculosis* (Myco; heat-killed) for the times indicated, and cytokine expression was determined as described in Figure 2B. Probes specific for the housekeeping genes GAPDH and ribosomal protein L32 served as controls for each sample.

or V_AE. A number of putative GATA and Ikaros binding sites are present within both CNS-1 and V_AE (Figure 5A). As shown by ChIP analysis in Figure 5B, GATA1, GATA2, and Ikaros were found to associate with the CNS-1 and V_AE elements. Binding to V_AE was generally more variable compared with CNS-1. The demonstration that GATA and Ikaros associated with these sites in nonstimulated MCs suggests that they are involved in regulating the Th2 locus.

Ikaros regulates IL-4 expression in MCs. We have previously shown that GATA1 and GATA2, like GATA3 in Th2 cells, are positive regulators of *Il4* expression in MCs. Association of these factors with the IE is critical for intron acetylation and subsequent transcription (29–31). In contrast to the sole positive actions of GATA family members, Ikaros can act as either an activator or a suppressor of transcription, activating expression of CD8 α in T cells and suppressing λ 5 expression in B cells (32, 33). To investigate the effect of Ikaros on *Il4* expression, BMMCs were derived from (B6 \times 129) F1 mice containing a targeted deletion of Ikaros (Ikaros^{null}) and their wild-type littermates (Ikaros^{+/+}) using standard differentiation culture conditions. RT-PCR assays revealed that Ikaros^{+/+} MCs expressed a variety of Ikaros isoforms and confirmed the absence of Ikaros expression in Ikaros^{null} MCs (Figure 6A). Like Ikaros^{+/+}, Ikaros^{null} MCs were c-kit^{hi} and Fc ϵ RI⁺ and contained the characteristic metachromatic granules (Figure 6B and data not shown), indicating that there were no defects in the ability to generate MCs from bone marrow. Although basal expression of Fc ϵ RI was consistently lower in Ikaros^{null} cells, treatment with monomeric IgE resulted in identical increases in receptor expression, confirming that downstream Fc ϵ RI-signaling pathways were intact (Figure 6B) (34). Ikaros^{null} BMMCs demonstrated elevated and more prolonged expression of *Il4* in response to Fc ϵ RI cross-linking compared with Ikaros^{+/+} MCs (Figure 6, C and D). To corroborate these findings at the protein level, activation-induced IL-4 secretion was measured in both groups (Figure 6E). IL-4 was detected in supernatants from Ikaros^{null} BMMCs 22 hours after Fc ϵ RI cross-linking and TLR2-mediated stimulation, but not in Ikaros^{+/+} BMMCs. TNF- α secretion was measured as a positive control for activation in both groups. While Ikaros^{null} BMMC expressed lower levels of IL-5 mRNA, expression of *Il13*, as well as *Il6* (a cytokine gene encoded outside of the Th2 locus), was not appreciably affected. Transcription of *Rad50*, a gene located within the Th2 locus but expressed constitutively in most cell types and under separate control than *Il4*, *Il5*, and *Il13*, was not affected in resting Ikaros^{null} BMMCs (Figure 6F). Taken together with the evidence of in vivo Ikaros binding to *Il4* regulatory elements, these data demonstrate that Ikaros is specifically involved in regulation of the *Il4* locus in MCs.

Ikaros controls Il4 expression at the chromatin level. To our knowledge, a direct mechanism by which Ikaros exerts its influence on the CD8 α and λ 5 loci has not been described previously (32, 33). The increased *Il4* expression observed in Ikaros^{null} BMMCs could be indicative of an overall suppressive effect on chromatin acces-

a strong transcriptional response in T cells, and V_AE is essential for optimal MC *Il4* expression (11, 13, 28). In addition, accessibility at both these sites is a key determinant in regulating the potential for high versus low IL-4 expression in T cells (27). Thus the potential for MC *Il4* expression correlated with a unique pattern of chromatin accessibility at several sites within the Th2 locus.

GATA and Ikaros family members associate with CNS-1 and the V_AE in vivo. The mechanism regulating *Il4* locus accessibility in MCs as well as T cells is poorly understood. Our finding that the acetylation state of *cis*-regulatory elements CNS-1 and V_AE is commensurate with *Il4* expression potential warranted a further investigation into the factors regulating these sites in MCs. No base pair polymorphisms were detected within these elements, indicating that strain-specific differences in *Il4* transcription are not due to alterations in transactivating factor binding sites within CNS-1

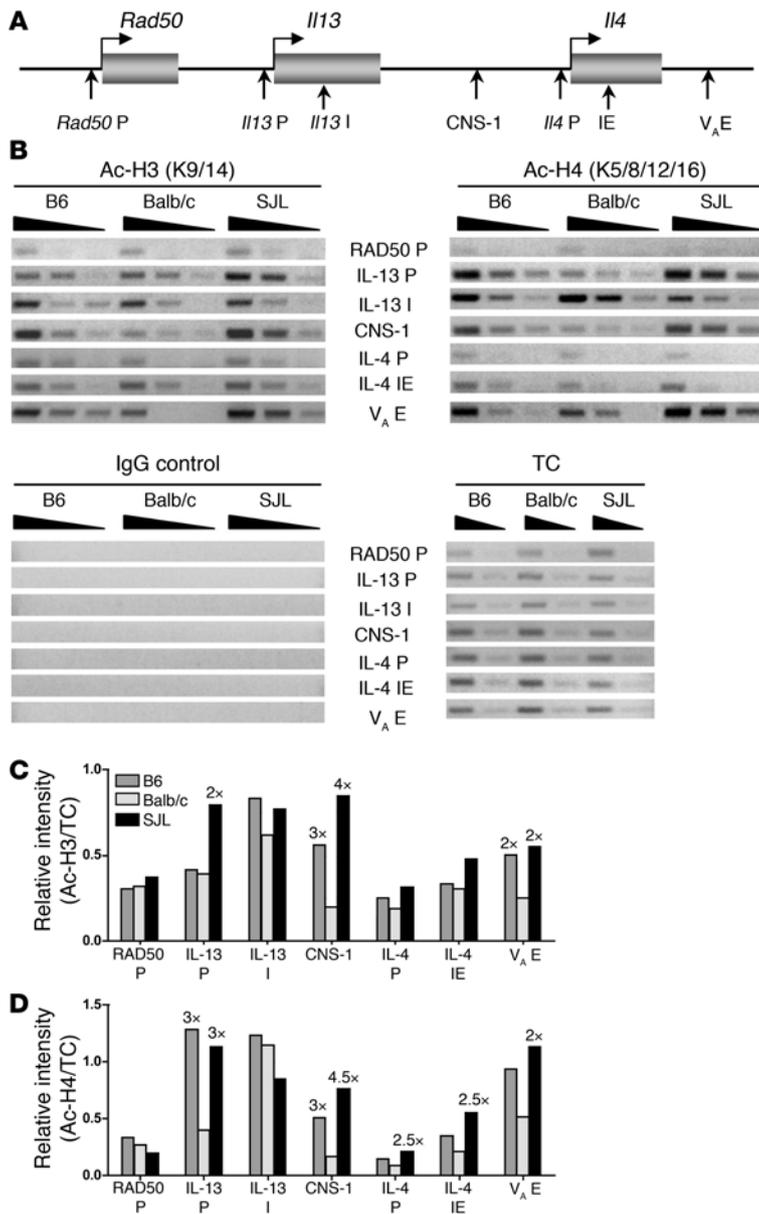


Figure 4

Histone acetylation at the Th2 locus in MCs correlates with strain-dependent differences in *Il4* expression. (A) Diagram of the murine Th2 locus showing a subset of defined regulatory elements. P, promoter; I, intron. (B) The relative levels of histone H3 and H4 acetylation (Ac-H3 and Ac-H4, respectively) at Th2 regulatory elements in resting BMMC were analyzed by ChIP assays. Antibodies specific to diacetylated histone H3 (lysine residues 9 and 14 [K9/14]) or tetraacetylated histone H4 (K5/8/12/16) were used. Three-fold dilutions of IP samples were used in PCR reactions, whereas 1:10 and 1:50 dilutions were used for PCR amplification of total chromatin input (TC). Data are representative of 4 experiments using independently derived BMMC cultures. (C and D) Data was quantified using NIH Image — relative intensity was calculated as the ratio of IP template to total chromatin input. Relative intensities at least 2-fold greater than that of Balb/c samples are shown above bars (2x, 3x, etc.).

the type and magnitude of a T cell response through the elaboration of immunoregulatory cytokines or expression of co-stimulatory molecules (35, 36). Most data supporting such a role have come from in vitro studies using long-term T cell and MC lines (35). An underlying hypothesis of the present investigation was that strain-specific variations in MC *Il-4* expression contribute to the genetically determined heterogeneity in Th responses. Several results of our study support this hypothesis. First, we showed that there were intrinsic differences in the ability of BMMCs, but not Th2 cells, derived from the B6, Balb/c, and SJL backgrounds to express *Il-4*. These differences were independent of the cell activation mode and correlated with distinct patterns of basal chromatin accessibility at the Th2 locus.

How could a variable pattern of accessibility within the Th2 locus be achieved that would give rise to distinct potentials for *Il4* expression? A clue is provided by the observation that GATA and Ikaros, factors with apparent opposing activity at the *Il4* locus, bound in vivo to regulatory elements of the Th2 locus. Notably, members of both of these families of transcription factors impact the development of specific cell lineages through their ability to modulate chromatin accessibility (37–39). In Th2 cells, GATA3 is essential for Th2 differentiation and the events associated with *Il4* locus opening (40). This factor is thought to create and maintain an open chromatin state in resting T cells (41), “poising” the locus for the interaction of inducible factors such as nuclear factor of activated T cells (NFAT) with their cognate sites upon cell activation (42–44). GATA3 enhances gene expression by increasing histone acetylation, presumably through the recruitment of CBP/p300-like proteins containing histone acetyltransferase activity. GATA3 can also antagonize the recruitment of DNA methyltransferase-1 and methyl CpG-binding domain protein-2 to CNS-1 in Th2 cells, 2 factors that would otherwise mediate repression of locus accessibility (45, 46). In MCs, GATA1 and GATA2 appear to play an analogous role to GATA3. However, while GATA2 is absolutely required for MC differentiation, a more predominant role in regulating gene expression in mature MCs has been assigned to GATA1 (39, 47). Both factors associate with the *Il4* IE in resting MCs and are necessary for main-

sibility at the Th2 locus. Ikaros can associate with the higher order nucleosome remodeler and deacetylase (NuRD) complex containing histone deacetylase 1 (HDAC1) and HDAC2. Thus if Ikaros is acting to recruit HDAC activity to the *Il4* locus, the lack of Ikaros expression was predicted to result in higher histone acetylation levels within the Th2 locus. As expected, acetylation of histones H3 and H4 was increased at most of the Th2 locus regulatory elements examined in Ikaros^{null} BMMCs compared with Ikaros^{+/+} BMMCs (Figure 7). Relative levels of histone acetylation at the *Gapdh* promoter were equivalent in both cell populations (data not shown). Thus Ikaros regulated the potential for *Il4* expression in MCs by reducing chromatin accessibility at the Th2 locus.

Discussion

It is well accepted that cells of the innate immune system can profoundly influence the character of the adaptive immune response. There has been much speculation that MCs in particular can affect



A

CNS-1

99971 **IK1** **GATA**
GAGCAGGGCTGGGGGAGTTTCTTAGGCCCTCTGCGAGGGGATCACTTTCCACTCGAGATGGCACGTGGAGGGGGAGGAAGA

100053 **IK2**
GAGAGCAACAGAAAGTGAGGTCGTAAGTAGAAAATTGCTTCTGGGATTTTCGAGTGGCTTCGTTCATGGGGCCCTCCCTGGCTG

100135 **IK3** **IK3.1**
CCGAGAAATCAGTTGATCTGGGAAAGTTTCGTTGCAAGCCATGCGCTCTGTTCGCTTTTGGGTGGAGCCGAGAAATGAATGAAG

GATA **IK4**
ATAATGAGGCCTCGTGACTTTTAGTTTTACAGTGAGAGGGGGCACGTGGGGGAAGGTGGCTATGGACACAGCTCTAGGCC

100299 **IK4.1**
TGACCAGCCTAAAGTGCGGGACCAGACAACCGGGGT**TCCCAT**GGTCAGGACACTCAGCCTGGTGCCTGGCCAGCAGCACCT

GATA
GCCACCATCTCAGAGGAGAGGGGAAACAGACCTGGTACCCACTGACCAGCTGCCTGCAAGGGGAAAGGCCAGGCTGCAGC

100463 **GATA** **GATA**
GTAATGTGACAGTGGATGGCTTAATTAGCCCCATCTCTCCTAGCTGTCTCAGACACCATTGTCTGACATAGGCCTAGTCTCT

V_AE

80161 **GATA** **GATA**
GGAAAAAATGTTTTTTTTCTCAATCTGGCTTTATTTAACATAATCTCCAACACTACATCTACTTTCTGTAAACATGACTTCAT

80242 **GATA** **GATA** **GATA**
TCTTCACGCCTAAGCACAACCTCCATTGTGTCTATGCACAACATATTCTTTATCTAGTTATCTGTTGATGCCTTGGTTCCATA

80324 **GATA**
TATTCATGACTGTGCAGTGCCACAATGATTGTGAATGTACAAGCATCTCTGTTGGGTTCTCAGTCCAACAGAGATATAATAT

80406
TTAATTCTTCAGAACCCTCCATACAGTTCTGTCAGTAGCTGCCCCAGCTTTCTTTGCCACTAGCAATGTTAAGTGCCTGG

80488 **GATA**
TGCCCTCTTGAGTATATCTTGGGG**TTGGGGAGATGGCT**CAGTGGGGCAAGAACACTTACTAAGGTGGCATCAGGACCTGAAT

Ikaros

GATA
TCACATCTCCAGCACTCATC

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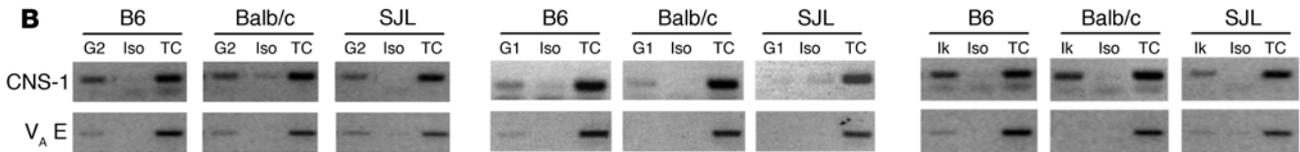


Figure 5

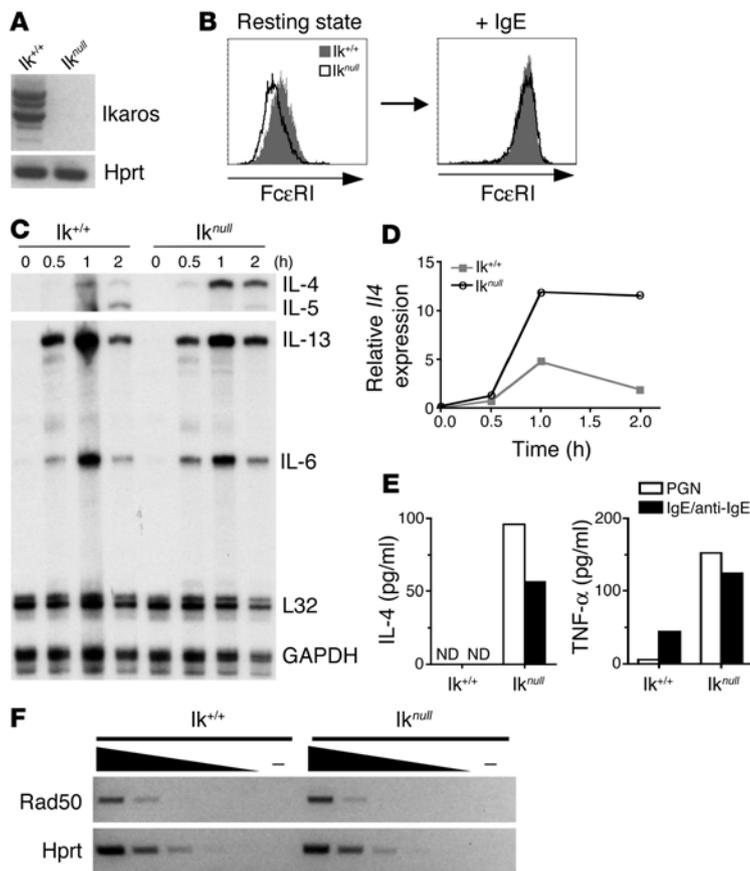
GATA1, GATA2, and Ikaros associate with the *cis*-regulatory elements CNS-1 and V_AE in vivo. (A) Genomic sequences of murine CNS-1 and V_AE within the Th2 locus. Sequences were analyzed using TFSEARCH (62), and putative binding motifs were identified. (B) GATA2, GATA1, and Ikaros proteins associated with CNS-1 and V_AE. Chromatin samples isolated from resting B6, Balb/c, and SJL BMMCs were used for ChIP analyses using GATA1-, GATA2- and Ikaros-specific antibodies (G1, G2, and Ik, respectively). All samples within a particular set (antibodies, isotype, and total chromatin control) were analyzed at the same time using identical PCR conditions and electrophoresed on the same agarose gel.

taining a demethylated DNA and acetylated histone chromatin state required for transcription (29–31). Based on these previous studies, GATA family members are considered positive regulators of chromatin accessibility in both cell types.

In contrast, our data indicated that Ikaros exerts a negative influence on expression from the Th2 locus. Ikaros was first characterized as an integral component of chromatin remodeling complexes in cells of the lymphoid lineage. Mice containing a targeted deletion in *Ikaros* have profound defects in both B and T cell development (37). The defined molecular targets of Ikaros have been limited to only 3 genes expressed during lymphoid cell development: CD8 α , TdT, and λ 5 (32, 33, 48). Our study provides what we believe to be the first direct evidence that Ikaros is also involved in *Il4* regulation. Not only did Ikaros associate with *cis*-regulatory elements of *Il4*, but Ikaros^{null} MCs derived from (B6 \times 129) F1 mice had dysregulated *Il4* expression. The increased IL-4 mRNA and

protein expression as well as elevated histone acetylation at CNS-1, V_AE, and other sites observed in Ikaros^{null} MCs confirms that this factor exerts a suppressive influence on *Il4*.

Ikaros was initially implicated in gene repression by virtue of its association with epigenetically silenced genes in pericentromeric heterochromatin (49). In Th1 cells, CNS-1 was essential for the localization of *Il4* to this nuclear compartment, an event that is associated with transcriptional blockade (16). It is hypothesized that Ikaros mediates this process through direct effects on the CNS-1 element. As IL-4 mRNA expression was still observed in Ikaros^{+/+} MCs, it is likely that Ikaros acts to modulate rather than completely suppress overall potential for transcription. The negative role of Ikaros in chromatin remodeling is also assumed to be due, in part, to its ability to form complexes with NuRD (50). Ikaros's repressive action on *Il4* transcription in MCs may be the result of targeting of NuRD-associated HDAC activity to relevant *cis*-regulatory elements.

**Figure 6**

Ikaros suppresses *Il4* transcription in MCs. (A) Resting BMMC express a number of Ikaros isoforms. Total RNA was analyzed by RT-PCR for the expression of Ikaros using primers specific for exons 1 and 7. Expression of the housekeeping gene *Hprt* was included as a positive control. Absence of Ikaros mRNA expression in *Ikaros*^{null} BMMCs was also confirmed. (B) Surface expression of Fc ϵ RI on BMMCs derived from *Ikaros*^{+/+} and *Ikaros*^{null} mice. Cells were left untreated or were cultured with monomeric IgE (5 μ g/ml) overnight. Fc ϵ RI expression was analyzed by flow cytometry. (C and D) *Ikaros*^{+/+} and *Ikaros*^{null} BMMCs were stimulated by Fc ϵ RI cross-linking, and cytokine mRNA expression was analyzed by ribonuclease protection assay. IL-4 mRNA expression was quantified as in Figure 2C. Data are representative of 4 independently derived BMMC cultures. (E) Cytokine secretion by activated *Ikaros*^{+/+} and *Ikaros*^{null} BMMCs measured 22 hours after activation with peptidoglycan or Fc ϵ RI cross-linking (IgE/anti-IgE). Mean of 2 independent experiments is shown. ND, not detected. (F) Rad50 and *Hprt* mRNA expression was analyzed from resting *Ikaros*^{+/+} and *Ikaros*^{null} BMMCs by semiquantitative RT-PCR.

progenitors. Further accessibility leading to a strong *Il4* transcriptional response may be mediated by GATA1.

The observed variability in chromatin accessibility between strains may be inherent to very early MC precursors and/or result from variations in signals downstream of IL-3 and SCF during in vivo and/or in vitro differentiation. Whereas signals delivered by IL-4 and antigens instruct locus opening in Th2 cells, MC growth factors IL-3 and SCF likely work in parallel fashion to affect remodeling during differentiation. Of note, both

GATA1 and GATA2 have been implicated as essential transactivators downstream of IL-3 signaling (54). Continued association of Ikaros with relevant elements may allow for a more adaptable locus that can readily alter chromatin accessibility depending on the cells' local microenvironment. This model does not purport that the binding of Ikaros or GATA at individual sites alone can control relative accessibility. Rather, it is the sum of interactions at multiple Th2 locus elements that is likely to dictate the high versus low expression potential. Other possible mechanisms contributing to genetically determined variability in accessibility at this locus could include differences in specific binding patterns of these and other factors, altered ratios of Ikaros to GATA binding, or expression of unique Ikaros isoforms that have distinct functional capabilities. It is also predicted that the interplay between the actions of GATA3 and Ikaros may influence chromatin accessibility in naive and differentiating CD4 T cells, leading to the extremes in expression potential in Th1/2 cells.

Using a MC knock-in model, we also demonstrated that MCs were required for an optimal antigen-specific IFN- γ response by CD4 T cells. More importantly, MC-derived IL-4 was a critical cytokine for this response, supporting the idea that the variable MC IL-4 response in B6 versus Balb/c mice is physiologically relevant. We propose that in certain immune settings, the relatively high IL-4 response of B6 MCs leads to the Th1-dominated responses characteristic of this strain. Conversely, Balb/c MCs do not express sufficient IL-4 to drive a strong Th1 response and default to the Th2 pathway. While this interpretation appears somewhat paradoxical with respect to the conventional Th1/Th2 cell paradigm, these

Our data correlating chromatin accessibility with IL-4 expression potential in MCs reveals a possible parallel to mechanisms governing the regulation of this gene in Th cells. Several studies have documented that transcription from the *Il4* locus in Th2 cells is a stochastic process whereby the probability of expression from either allele is associated with the degree of chromatin accessibility (27, 51, 52). It is hypothesized that this process allows for a more flexible means of regulation and adds an additional level of control to cytokine gene expression. In Th cells, CNS-1 and V_AE are the currently defined targets of probabilistic control, making them key determinants in regulating *Il4* expression potential (27). These same sites appeared to influence differential potential for MC *Il4* expression. It will be of interest to determine whether there is variability at the single-cell level within MCs of a particular genetic background, as in Th2 cells (51).

Based on our data, we propose a model whereby the integration of GATA and Ikaros signals, which can exert opposing effects on chromatin conformation, are responsible for distinct levels of accessibility at the Th2 locus (Figure 8). There are several possible scenarios. For example, Ikaros may be associated with relevant *Il4* cis-regulatory elements in early myeloid-lymphoid progenitors and silence expression in these cells. During MC development, lineage-specific transcription factors such as GATA family members function as "pioneering" proteins to decompact the condensed chromatin and promote a cell lineage-specific gene expression profile (53). The extent of GATA pioneering activity may be dependent on relative expression levels or activation states. Thus GATA2 may act as a pioneering factor for initial opening of the *Il4* locus in early MC

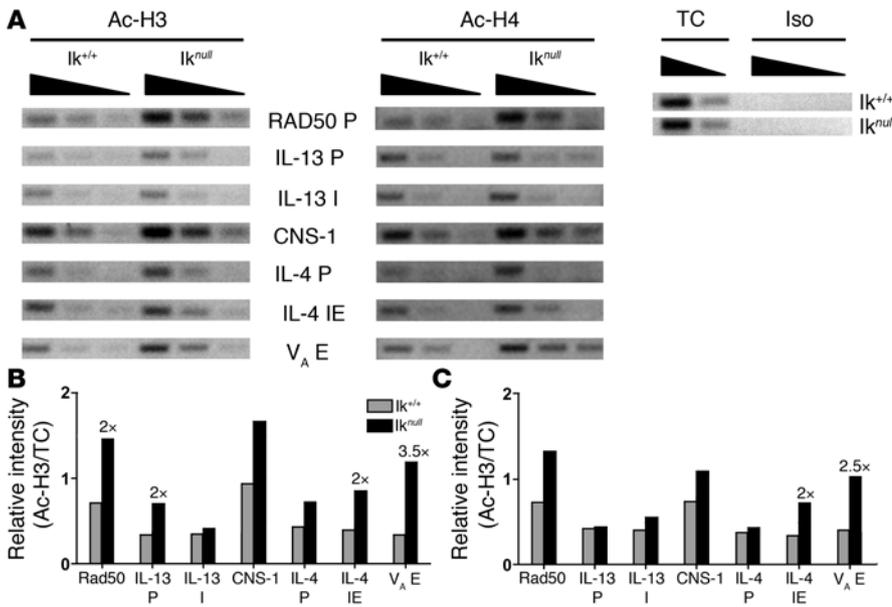


Figure 7

Ikaros regulates chromatin accessibility of the Th2 locus. (A) Th2 locus accessibility in Ikaros^{null} BMMC was assessed by H3 and H4 acetylation using ChIP as in Figure 4. Representative data for total chromatin input and isotype control is shown (CNS-1 primer set). Three-fold dilutions of IP samples were performed, whereas total chromatin input was analyzed using 1:10 and 1:50 dilutions. Data are representative of 3 assays using 2 independently derived MC cultures. (B and C) Relative acetylation of histones H3 (B) and H4 (C) were calculated as the ratio of IP template to total chromatin input. Relative intensities at least 2-fold greater than that of Ikaros^{+/+} samples are shown above the bars.

data highlight the importance of the spatial and temporal expression of IL-4 in the event of a response *in vivo*. They also support a model proposed by Biedermann et al., who demonstrated that the duration of IL-4 treatment during *L. major* infection of Balb/c mice directs the character of the Th response (2). When present only during DC maturation, IL-4 induces an IL-12-producing DC1 phenotype promoting Th1 differentiation. If IL-4 is administered for longer periods and is present during T cell priming in the secondary lymphoid organs, a typical Th2-dominated response is generated. The ability of MCs to express IL-4 in response to a variety of stimuli and their close proximity to DCs in many tissues including the skin make them likely candidates to modulate DC function in inflammatory settings (55).

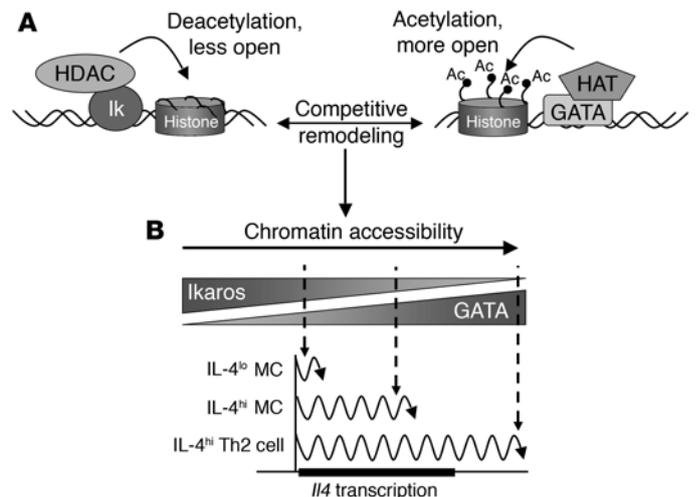
There is some indirect evidence to support the idea that genes regulating IL-4 expression potential and chromatin accessibility influence disease heterogeneity. Genes controlling susceptibility to and IL-4 expression during *L. major* infection map to a number of loci, including *L. major response 6 (Lmr6)*. Located at the near end of chromosome 11, *Ikaros* is found within *Lmr6* and is a candi-

date susceptibility gene (56, 57). Our identification of Ikaros as a gene controlling Th2 cytokine production strengthens these findings. Teuscher and colleagues have also implicated this region in EAE disease severity (58). Further investigations using mice with cell-specific targeted deletions of Ikaros may yield a better understanding of the role of Ikaros in these disease settings.

In summary, although the role of Ikaros is well defined in hematopoietic cell development, little is known about its function in the regulation of genes expressed during immune responses. Our finding that Ikaros was intricately involved in regulation of the Th2 locus in MCs reveals what we believe to be a novel and important role for Ikaros in regulating genes of mature effector cells. Our data also expand our current understanding of the molecular basis for genetically influenced heterogeneity in immune responses and reinforce the idea that MCs are critical regulatory/amplifying components of type 1 immune responses. Such information is critical for refining strategies for changing the class of an immune response, particularly in the setting of vaccine development.

Figure 8

GATA and Ikaros collaborate at the Th2 locus to govern *Ii4* expression potential. (A) Model proposing that Ikaros and GATA proteins — which can associate with Th2 locus *cis*-regulatory elements concurrently — competitively remodel the local histone state via the recruitment of HDAC or histone acetyltransferase (HAT) activity, respectively. (B) The combination of Ikaros and GATA binding to multiple sites within the Th2 locus, in addition to each factors' inherent ability to recruit suppressive (e.g., HDAC) versus activating (e.g., HAT) complexes, sets the potential for high, intermediate, or low *Ii4* expression in MCs derived from genetically disparate strains. In Th2 cells, relatively high GATA3 expression leads to efficient opening of the Th2 locus, resulting in a permissive chromatin state.





Methods

Mice. Balb/cj, SJL/J, B6, and B6-*Il4^{tm1Nnt}/J* female mice (5–6 weeks old) were purchased from The Jackson Laboratory. Female MC-deficient W/W^v mice and their congenic littermate controls (WBB6-F1/J-Kit⁺-Kit⁺) were obtained at 3 to 5 weeks of age. Ikaros^{null} mice (B6 × Sv129) were originally generated by K. Georgopoulos (Massachusetts General Hospital, Charlestown, Massachusetts, USA) (37). Mice were housed in the animal care facilities at Emory University and Northwestern University. All animal experiments were approved by the Institutional Animal Care and Use Committee at Emory University and the Animal Care and Use Committee at Northwestern University.

Generation of BMMCs and Th2 cells. Bone marrow was harvested from 5- to 6-week-old mice and differentiated with recombinant IL-3 (rIL-3; 5 ng/ml) and SCF (12.5 ng/ml) in complete RPMI (15% heat-inactivated FBS, 2 mM glutamine, 1% penicillin-streptomycin, 1 mM sodium pyruvate, and 50 μM 2-β-ME). At 6–8 weeks of culture, these populations were >95% MCs as assessed by c-kit and FcεRI expression and toluidine blue staining. Th2 cells were differentiated in vitro with plate-bound anti-CD28 and anti-TCRβ as previously described (44).

For optimal cytokine expression, MCs were cultured at 5 × 10⁵ cells/ml in 5 ng/ml rIL-3 (termed day 0) and stimulated on day 3. FcεRI cross-linking was accomplished using a previously described mode of IgE/anti-IgE activation (59). RNA was obtained using RNA-STAT60 (Tel-Test Inc.). For activation via TLR agonists, cells were suspended at 5 × 10⁵ cells/ml in complete rIL-3-containing RPMI on day 3 and stimulated with 50 ng/ml LPS or 100 μg/ml sonicated peptidoglycan (Sigma-Aldrich) or heat-killed *M. tuberculosis* strain H37RA (BD Diagnostics). Th2 cells were activated for 24 hours by plate-bound anti-TCRβ (0.5 μg/ml). For cytokine protein analysis, activated BMMCs were cultured at 2 × 10⁶ cells/well in 200 μl complete DMEM supplemented with IL-3. Culture supernatants were collected at 22 hours, and cytokine levels were assayed using LiquiChip Workstation (Qiagen) as previously described (22).

Ribonuclease protection assays. Assays were performed per the manufacturer's instructions (RiboQuant Multi-Probe RNase Protection Assay System; BD Biosciences). Gels were visualized by autoradiography and/or quantified via phosphorimaging and ImageQuant software (GE HealthCare).

ChIP. Analysis of histone H3 and H4 acetylation and in vivo binding of GATA1/2 and Ikaros was performed using ChIP per the manufacturer's instructions (Upstate USA Inc.) and as previously described (29) using resting BMMCs. A polyclonal Ab to Ikaros (H-100) was obtained from Santa Cruz Biotechnologies Inc. Immunoprecipitates were resuspended in 50 μl of H₂O, and serial 3-fold dilutions of 1 μl template were used for each reaction. Sequences of primers used in PCR were as follows: Rad50 promoter forward, 5'-GTGCGAGCTTACGGTTGCTGGCTCGC-3'; Rad50 promoter reverse, 5'-AACCTCAAGCGGAAGCTGGGCAAG-3'; IL-13 promoter forward, 5'-TCCAACCATGCATTGCTTTGGTG-3'; IL-13 promoter reverse, 5'-TTTGTGGGCATTATCTGAAAACC-3'; IL-13 intron forward, 5'-GTGAGTAGCACACAGCCCCTCC-3'; IL-13 intron reverse, 5'-TGATAAACAGTGGTCGCCACTCC-3'; CNS-1 forward, 5'-TGATTCTC-GGCAGCCAGGGAGGGCC-3'; CNS-1 reverse, 5'-GGTGCCTGCGT-

CACCTCTGACCACAC-3'; IL-4 promoter forward, 5'-ACTCATTTTCCTTG-GTTTCAGC-3'; IL-4 promoter reverse, 5'-GATTTTTGTGCGATCCGTGG-3'; IL-4 intron forward, 5'-TCTGCTTGGACATCTCTCTTCCC-3'; IL-4 intron reverse, 5'-ACCACCCACAGGTCTTTGTTC-3'; V_αE forward, 5'-CAG-GTCCTGATGCCACCTTAGTAAG-3'; V_αE reverse, 5'-TTTAAATCCA-GATTCTGCATAGAATGG-3'. PCR conditions were 95°C denaturation, 60°C annealing, and 72°C extension for 30–36 cycles. PCR products were visualized with ethidium bromide on a 1.5% agarose gel.

RT-PCR. RNA was obtained from resting MCs as described above. RNA was reverse transcribed using oligo-dT and SuperScript II. The following primers, complementary to exon 1 and exon 7 sequences, were used to amplify Ikaros isoforms: Ik forward, 5'-GATAGATCTATGGATGTCGAT-GAGGGTCAAGAC-3'; Ik reverse, 5'-GATGAATCTTAGCTCAGGTGGTA-ACGATGCTC-3' (60); Hprt forward, 5'-GTTGGATACAGCCAGACTTT-GTTG-3'; Hprt reverse, 5'-GAGGGTAGGCTGGCCTATAGGCT-3'. TLR2 and TLR4, amplified for 35 cycles, were used as previously described (61).

Reconstitution and disease induction. Four-week-old W/W^v mice were reconstituted with 4 × 10⁶ BMMCs by i.v. transfer. Selective reconstitution of the MC compartment was confirmed by hematocrit, histology, and/or flow cytometry at >8 weeks after transfer (Supplemental Figure 1; available online with this article; doi:10.1172/JCI27227DS1; and data not shown). EAE was induced by s.c. injection of 300 μg MOG₃₅₋₅₅ emulsified in 5 mg/ml CFA and 250 ng pertussis toxin i.p. Mice were scored daily for paralysis as previously described (22).

Intracellular cytokine staining and flow cytometry. Preparation of CNS-infiltrating lymphocytes and in vitro restimulation of splenocytes with MOG₃₅₋₅₅ peptide was performed as described previously (22). Intracellular cytokine expression for IFN-γ expression was assayed using the Cytofix/Cytoperm Kit (BD Biosciences) and analyzed by flow cytometry.

Statistics. Statistical analysis was performed using GraphPad Prism software (version 4.0a). Repeated-measures ANOVA followed by Dunnett's multiple-comparison post-test was used for comparison of mean clinical scores in EAE experiments. Intracellular cytokine expression was analyzed by unpaired Student's *t* test. *P* values less than 0.05 were considered to be statistically significant.

Acknowledgments

This work was supported by NIH grant R01A047992 and grant RG3104 from the National Multiple Sclerosis Society (to M.A. Brown).

Received for publication October 26, 2005, and accepted in revised form February 28, 2006.

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