Deletion of a coordinate regulator of type 2 cytokine expression in mice

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Abstract

Mechanisms underlying the differentiation of stable T helper subsets will be important in understanding how discrete types of immunity develop in response to different pathogens. An evolutionarily conserved ~400 base pair non-coding sequence in the IL-4/IL-13 intergenic region, designated CNS-1, was deleted in mice. The capacity to develop Th2 cells was compromised in vitro and in vivo in the absence of CNS-1. Despite the profound effect in T cells, mast cells from CNS-1-deleted mice maintained their capacity to produce IL-4. A T cell-specific element critical for optimal expression of type 2 cytokines may represent evolution of a regulatory sequence exploited by adaptive immunity.

Introduction

Mechanisms by which naive T cells acquire their functional cytokine repertoire are critical to understanding how different types of antigenic challenge are met with discrete immune responses. Cytokines themselves are crucial in this process, and work to stabilize expression of certain cytokine genes while suppressing others, thus leading to the eventual development of polarized populations of effector cells best suited for different types of infectious organisms. The most prevalent polarized T helper cell subsets, Th1 cells characterized by IFN-γ production and Th2 cells characterized by IL-4 production, develop in response to the cytokines IL-12 and IL-4, respectively, as mediated by critical Stat transcription factors associated with their relevant cytokine receptors on naive T cells (1).

Recent studies suggest that cytokine genes might occupy transcriptionally permissive areas in the nucleus in naive T cells, and that Stat signals may serve to stabilize patterns of cytokine gene transcription initiated by signals transmitted by the antigen receptor (2). Stabilization of patterns of cytokine gene expression has been associated with structural changes in chromatin around expressed genes (3-6). Such changes may allow access of lineage-determining transcription factors such as GATA-3 for Th2 cells and T-bet for Th1 cells (7, 8). Indeed, a DNase I hypersensitivity site appearing during the differentiation of Th2 cells identified an enhancer element 3' of the IL-4 gene that selectively recruited GATA-3, thus serving to focus the more generally expressed NFAT1 transcription factors at the IL-4 promoter and enhancer sequences (9).

Elucidation of the rules that identify such critical regulatory elements will be fundamental to understanding cell fate decisions linked intricately with host responses in the immune system. In a screen for potential regulatory elements, ~1 Mb of human chromosome 5q31 and the orthologous region on mouse chromosome 11 were compared for conserved noncoding sequences (CNS). Using criteria associated with experimentally identified regulatory regions - >100 bp with >70% homology (10) - 91 elements were located within intergenic regions. At 401 bp, CNS-1 was the longest of these elements, and additional sequence comparison revealed substantial conservation across mammals, including mice, humans, cows, dogs and rabbits (11). The localization of CNS-1 between the IL-4 and IL-13 genes, cytokines coordinately expressed from individual alleles in appropriately activated T cells (12), suggested a potential regulatory role for CNS-1 in modulating cytokine expression. Indeed, using YAC transgenic mice containing integrated human cytokine genes and a loxP-flanked CNS-1 sequence, not only human IL-4 and IL-13, but also IL-5, which resides some 120 kb from IL-13 across the intervening RAD50 gene, were attenuated in expression from transgenic T cells in the absence of CNS-1 (11).

Using gene targeting in the mouse germline, we created CNS-1-deleted mice to test directly whether this element is involved in expression of the type 2 cytokines in vitro and in vivo using characterized antigenic challenges. The results not only establish CNS-1 as a crucial element for the expression of type 2 cytokines in T lymphocytes, but

validate inter-species genomic comparisons as a mechanism for finding critical elements involved in gene regulation linked to cell differentiation (13).

Results

Generation of CNS-1-deficient mice. CNS-1 was deleted in ES cells using homologous recombination and replacement with a loxP-flanked drug selection cassette (Fig. 1A). After obtaining germline transmission in targeted mice, animals were bred to mice that expressed cre recombinase in the germline, and offspring were screened to obtain mice with deletion of the selection cassette. Male heterozygous CNS-1-deleted mice were bred to female BALB/c mice and offspring were selected that were deleted for CNS-1, the neo selection cassette and absence of the cre transgene using Southern blotting (Fig. 1A, B, C). The net effect of targeting the CNS-1 element was replacement of 489 bp including the CNS-1 sequence with 255 bp of vector sequence containing one residual loxP site. Animals were intercrossed to obtain homozygous CNS-1-deleted mice, which were healthy and had no obvious phenotypic differences from wild-type animals. Analysis of thymus, lymph nodes and spleen revealed no impact of CNS-1 deletion on development or numbers of thymic or peripheral lymphoid cell populations or numbers (data not shown).

Type 2 cytokine expression in CNS-1-deleted CD4+ T cells. Highly purified, naive, CD4+ T cells (>99% $CD62L^{hi}$) from wild-type and heterozygous and homozygous CNS-1-deleted mice were labeled with the intracellular fluorochrome, carboxyfluorescein diacetate succinimidyl ester (CFSE), and were stimulated with antibodies against the

TCR and CD28 under Th1, neutral (no added polarizing cytokines), or Th2 conditions. After 4 days, cells progressed heterogeneously, in agreement with prior studies (2), with populations distributed from none through 6 divisions (Fig. 2A). Deletion of CNS-1 had no significant effects on cell progression through division.

Cells were assayed on day 4 using intracellular staining for expression of IL-4 and IFN-γ after re-stimulation using PMA/ionomycin. Under Th2 conditions, CNS-1-deleted cells demonstrated fewer IL-4-expressing cells in each cell division and less IL-4 production per cell, as assessed by the mean fluorescence intensity (MFI) (Fig. 2B). Heterozygous CNS-1-deleted mice had an intermediate phenotype. After 5 days of priming under Th2 conditions, CNS-1-deleted T cells produced not only less IL-4, but also less IL-5 and IL-13, as assessed by ELISA of culture supernatants 48 hrs after re-stimulation (Fig. 2C). Under neutral or Th1 conditions, CNS-1-deleted mice demonstrated no differences from wild-type T cells; IFN-γ levels were equivalent under either condition and IL-4 production was minimal (data not shown).

Activation of naive T cells using plate-bound anti-TCR and anti-CD28 antibodies results in rapid transcription of the IL-4 as assessed using quantitative kinetic PCR (2). As compared to wild-type CD4+ T cells, CNS-1-deleted cells demonstrated delay in the appearance of IL-4 transcripts (Fig. 3A). Although CNS-1-deleted cells increased IL-4 transcripts over the subsequent 20 hrs, a diminution in transcript abundance continued to be present; cells examined after 5 days in culture had similarly diminished IL-4 mRNA (Fig. 3B).

IL-4 expression from CNS-l-deleted mast cells. Mast cells produce IL-4 by distinct mechanisms as compared to T cells. Although Stat6, GATA3 and c-maf have each been demonstrated to be critical for the production of IL-4 by T cells, none of these factors is required in mast cells (14-16). To test requirements for CNS-1 in mast cell IL-4 production, bone marrow-derived mast cells from wild-type and homozygous CNS-1-deleted mice were stimulated with ionomycin and assayed for IL-4 production using ELISPOT assays. Under these conditions, no differences in numbers of IL-4-producing cells or amounts of IL-4 produced were demonstrated despite the ease with which differences were seen using CNS-1-deleted T cells stimulated using PMA/ionomycin (Fig. 4). Thus, at least under these conditions, mast cells, in contrast to T cells, do not require the CNS-1 element for optimal IL-4 expression.

Expression of type 2 cytokines in vivo in CNS-1-deleted mice. IL-4, and to a lesser extent, IL-13, mediates immunoglobulin isotype switching to IgE (17). When age-matched, littermate, wild-type, heterozygous and homozygous CNS-1-deleted mice were examined for baseline IgE, a direct correlation was apparent between the presence of CNS-1 and spontaneous serum IgE concentration, suggesting a role for this element in mediating type 2 cytokine expression in vivo (Fig. 5).

To explore further the effects of CNS-1 deletion in vivo, mice were infected with Nippostrongylus brasiliensis, a strong inducer of type 2 immune responses (18). After 8 days, lymphoid cells from the lung and mesenteric lymph nodes were purified and examined for IL-4 expression by intracellular cytokine staining. Whether collected from

tissue lung sites or lymph nodes, CD4+ T cells from CNS-1-deleted mice contained significantly fewer IL-4-producing cells with a smaller MFI per cell than did wild-type mice (Fig. 6).

Intratracheal challenge with extracts of the fungus Aspergillus fumigatus induces strong lung type 2 immune responses in mice, and has been used to model allergic airways diseases, such as asthma (19). Challenged mice develop enhanced airway reactivity to bronchoconstricting agents and develop elevations in serum IgE in association with infiltration of IL-4- and IL-5-producing CD4+ T cells into the lungs. Cohorts of age-matched mice in the designated groups were challenged with Aspergillus and analyzed 4 days after the final antigen dose. Both IL-4-producing and IL-5-producing CD4+ T cells were significantly attenuated in CNS-1-deleted mice (Fig. 7A). Bronchial reactivity to acetylcholine was less, although not statistically significant, and serum IgE was also diminished in CNS-1-deleted mice (Fig. 7B, C).

Leishmania major is an intracellular protozoan contained in the host by Th1 cells that produce IFN-γ. The capacity to clear parasites is abrogated by Th2 cells that produce IL-4. Various mouse strains produce a spectrum along a continuum from highly resistant Th1-associated responses to highly susceptible Th2 responses, with the degree of Th2 responsiveness inversely correlated with the capacity to clear the infection (20). Age-matched wild-type and CNS-1-deleted mice were infected in the hind footpads with L. major and the size of the footpad lesions followed over the subsequent 6 weeks. Footpads from CNS-1-deleted mice were significantly smaller than from wild-type mice,

and correlated directly with fewer numbers of parasites recovered from tissues (Fig. 8A, B). When assessed using ELISPOT assays, the popliteal lymph nodes draining the lesions contained significantly fewer IL-4-producing cells from CNS-1-deleted, as compared to wild-type, mice (Fig. 8C). Serum IgE was also attenuated (Fig. 8D).

Discussion

CNS-1 is a 401 bp element located in the IL-4 and IL-13 intergenic region that shares 89% homology between mice and humans (11). Deletion of this sequence resulted in no apparent effects on lymphoid development in mice. As assessed both in vitro and in vivo using three distinct antigenic challenges, however, CNS-1 was required for optimal expression of the type 2 cytokines. Although expression of IL-4 was investigated most thoroughly, both IL-13 and IL-5 were attenuated under various conditions. This study both validates an indirect approach using human YAC transgenes to investigate the role of CNS-1 in control of type 2 cytokine expression, and definitively implicates this region as an element mediating the expression of these cytokines in vitro and in vivo (11). The identification of this distant regulator of the type 2 cytokine loci will provide an important tool for understanding the coordinate expression of these cytokines that remains the cardinal characteristic of Th2 cells.

Extensive mutational analysis has linked cell- and activation-dependent expression of IL-4 to the proximal promoter element (21-23). Five NFAT binding sites, two associated with AP-1 family members, are required for inducible IL-4 expression in vitro (24-26). Although NFAT and AP-1 proteins are not Th2-specific, knock-out studies of NFATc1

and JunB have implicated these members of the respective protein families in the optimal differentiation of Th2 cells (27-29). Despite these findings, IL-4 promoter transgenic mice expressed the transgene at levels much lower than the endogenous IL-4 gene, implicating additional regulatory elements outside of the promoter (30). DNase I hypersensitivity analysis was used to identify a 3' IL-4 enhancer that served to recruit GATA-3, a Th2 lineage differentiation factor (7), during differentiation of Th2 cells in vitro. This distal enhancer increased IL-4 promoter-driven transcription approximately 2-fold in vitro, although no in vivo studies were performed (9). Using a transgenic approach, a mini-locus that included CNS-1 functioned as a strong enhancer for the IL-4 promoter, although Th2-specific expression required linkage with additional proximal elements (31).

Whereas deletion of CNS-1 caused a robust phenotype, secretion of type 2 cytokines was not ablated entirely. Whether this was due to the strong stimulus used - cross-linking antibodies to the TCR and CD28 - or reveals the presence of alternate, but less efficient, mechanisms for activation of these genes will require further study. As demonstrated here, no requirement for CNS-1 was revealed in mast cells using similar conditions for activating IL-4 expression. Three independent models of antigen challenge, however, verified the requirement of CNS-1 for optimal Th2 differentiation. Nippostrongylus infection and inhalational challenge with Aspergillus represent strong stimuli for Th2 cell differentiation and, in each case, deficiency of CNS-1 was associated with attenuation in the numbers of cells expressing type 2 cytokines. Additionally, downstream targets of

these cytokines, such as class switching to IgE, were clearly impacted by the lack of this regulatory sequence. Like the in vitro data, however, type 2 cytokine responses were attenuated, but not entirely absent. This likely reflects the lack of requirement for CNS-1 by other cell types, such as mast cells, together with the capacity of T cells to express these cytokines, albeit less efficiently, in the absence of CNS-1. Similar observations have been made using Stat6-deficient cells; although IL-4 expression was substantially attenuated, some cells stochastically progressed through the requirement for Stat6 and subsequently achieved stable IL-4 expression (32). In the Leishmania model, a CD4-dependent system in which outcome is linked tightly with Th development, the absence of CNS-1 was associated with a robust phenotype that enhanced the capacity of mice to resolve this intracellular infection. By this paradigm, pharmacologic targeting of CNS-1 to block its function might be predicted to ameliorate diseases such as allergy and asthma characterized by an exuberant Th2 response.

The sequence conservation of CNS-1 together with its single position in the human genome as assessed using Southern blot analysis are features shared by experimentally identified distant regulatory elements (10). As shown here, CNS-1 was necessary for optimal expression of IL-4 during differentiation of Th2 cells in vitro. In the absence of CNS-1, IL-4 transcription was delayed and protein accumulation was substantially attenuated. Decreased expression of IL-13 and IL-5 also occurred in the absence of CNS-1, and likely represents a direct effect rather than a consequence of diminished IL-4 production, since exogenous IL-4 was present at high levels during Th2 priming in vitro

(Fig. 2). Of the three DNase I hypersensitivity sites located in the IL-4/IL-13 intergenic region, two were induced during the differentiation of Th2 cells and were encompassed in CNS-1 (3, 33). Ectopic expression of activated Stat6 or GATA-3 led to chromatin remodeling at the CNS-1 site, and GATA-3 was capable of binding to a sequence within one of the DNase I hypersensitivity regions (34, 35). Together with the data here demonstrating a requirement for CNS-1 to optimize the initial transcription of IL-4 and to stabilize the differentiation of Th2 cells in vitro and in vivo, we hypothesize that CNS-1 functions to regulate the accessibility of cell-specific proteins that coordinate stable transcription of these genes. Consistent with this interpretation, expression of reporter genes from a mini-gene locus containing CNS-1 with additional IL-4 promoter/enhancer elements was enhanced by the over-expression of GATA-3 (31). The demonstration of an intermediate phenotype in heterozygous CNS-1-deleted cells suggests a a cis-acting effect of CNS-1 (Fig. 2B, Fig. 5), and would be consistent with the coordinate regulation of the linked IL-4, IL-13 and IL-5 genes revealed in analysis of T helper clones (12).

The mechanisms by which distant elements function to regulate gene expression remain incompletely defined. The globin gene locus control region (LCR), identified as a series of DNase I hypersensitivity sites 5' of the globin genes, is crucial in regulating the developmental expression of these genes in erythroid cells. Although various models for 'looping' or 'linking' have been proposed, each envisions a physical reorganization that brings the LCR into proximity of the appropriately transcribed globin gene by a mechanism coupling erythroid lineage-specific transcription factors to generally

expressed chromatin-modifying enzymes that regulate gene accessibility and transcriptional competence (36). Similar observations have been made in studies of immunoglobulin heavy and light chain enhancers; B cell lineage- and stage-specific regulation of gene expression was linked tightly with demethylation of the immunoglobulin locus (37-39). T cell receptor α, β and δ gene enhancer elements similarly influence accessibility by the recombination machinery (40, 41). The location of CNS-1 at a Th2 condition-dependent DNase I hypersensitivity site (3), its ability to bind Th2-dependent DNA binding proteins (33), and, as shown here, its requirement for optimal transcription and stabilization of the type 2 cytokine genes in vitro and in vivo, suggests that CNS-1 likely functions to recruit general chromatin remodeling enzymes to these genes.

Intriguingly, despite a requirement for CNS-1 in T cells, CNS-1-deficient mast cells remained no different than wild-type mast cells in their capacity to produce IL-4 under the conditions used. Factors required for T cell expression of IL-4 - including GATA-3, c-maf and Stat6 - are either not expressed or not necessary for IL-4 expression in mast cells (14-16). Instead, an element in the second intron of the IL-4 gene functions both to enhance transcription and to influence methylation of the IL-4 gene (42). The intronic enhancer serves to bind mast cell-specific factors - PU.1, GATA-1 and -2 and possibly Stat5a/5b - and is required for IL-4 expression in mast cells but not T cells. Unlike mast cells, T cells have the capacity to differentiate into long-lived memory cells that maintain epigenetically modified cytokine effector programs, an ability that underlies adaptive, in

contrast to innate, immunity. Recent studies have demonstrated physical repositioning of cytokine genes in the nucleus that accompanies Th subset differentiation (2). Further study will be required to establish whether CNS-1 regulation has evolved to confer in T cells long-term modification of the locus that might not be necessary in cells of the innate immune system.

Materials and Methods

Generation of CNS-1 deficient mice. The targeting construct was amplified from 129/SvJ BAC 111-181 DNA from California Institute of Technology library CitbCJ7 (11) using primers for the two homology arms as follows: CNS-1SA forward 5'-TTACCCAGGTCCCCTAACTGG-3' and CNS-1SA reverse 5'-ATGGACACAGCTCTAGGCCTG-3 for the 1.8 kb short arm, and CNS-1LA forward 5'-CTGGCTGATTCCTAGCCCTAC-3' and CNS-1LA reverse 5'-GCACTTAGCCTGAGTCACCTG-3' for the 5.1 kb long arm. The targeting vector pPN2T (43) was modified by replacing the existing neomycin selection cassette with a neomycin selection cassette flanked by loxP sites from ploxPneo-1 (44). The resulting targeting vector, ploxPN2T, also contains a tandem array of thymidine kinase expression cassettes for counter-selection. The two homology arms were inserted into ploxPN2T on either side of the loxP-flanked neomycin cassette thereby replacing the 489 bp CNS-1 element with the loxP-flanked neomycin cassette upon correct targeting. 129/SvJ ES cells (Genome Systems) were electroporated with 20 μg of the NotI linearized targeting construct and subsequently selected in 200 μg/ml G418 and 0.5 μg/ml FIAU for 8 days.

Individual clones were isolated, expanded and screened by Southern blot (Fig. 1, probe b). The 367 bp probe b was amplified by PCR from BAC 111-181 DNA using the following primers: CNS-1b forward 5'-CAGGTGACTCAGGCTAAGTGC-3' and CNS-1b reverse 5'- GTCCCAGACTCCAAGTCTTCC-3'. Targeted clones were injected into C57BL/6 blastocysts and chimeric males were bred to C57BL/6 females. Offspring were tested for germline transmission by Southern blot using probe b and heterozygous animals were bred to mice expressing germline cre recombinase (45). Offspring were tested for deletion of the neomycin gene by Southern blot using probe c (Fig. 1). The 218 bp probe c was amplified by PCR from BAC 111-181 DNA using the following primers: CNS-1c forward 5'-GACACTTGTCCACACACCCTC-3' and CNS-1c reverse 5'-CACCTGAGACTTAGCCCAAGC-3'. Heterozygous CNS-1-deleted mice containing a deleted neo-cassette were bred to BALB/c females and offspring were screened by Southern blot and selected for the absence of CNS-1, the neo-cassette and the cre transgene. These mice were intercrossed in order to obtain homozygous animals.

T cell purification and activation. Naive, small, $CD62L^{hi}$, CD4+ T cells were purified to >99% using flow cytometry (MoFlo Multi-Laser; Cytomation, Fort Collins, CO) as described (46). Where indicated, cells were pre-incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) as described (2). Purified T cells were activated under neutral conditions in vitro using 10^6 CD4+ T with 5 x 10^6 irradiated antigen presenting cells (APC) prepared from TCR-Cα-deficient mice (47), and monoclonal antibodies to TCRβ and CD28 with 50 U/ml recombinant human

IL-2 as described (46). Th1 conditions included recombinant murine IL-12 (5 ng/ml) and anti-IL-4 antibody (11B11, 20 μg/ml). Th2 conditions included recombinant murine IL-4 (50 ng/ml) and anti-IFN-γ antibody (XMG1.2, 50 μg/ml). T cells activated in the absence of APC were stimulated using tissue culture plates pre-coated with anti-TCRβ and anti-CD28 as described (46).

Mast cell purification and activation. Bone marrow cells were flushed from the femurs of CNS-1-deleted and age-matched littermates and c-kit-expressing cells (PharMingen, San Diego, CA) were purified using flow cytometry. Sorted cells were cultured in standard tissue culture medium supplemented with 1 μg/ml recombinant murine IL-3 (R&D Systems, Minneapolis, MN). After 5-10 days, the majority of cells had the short cytoplasmic projections, indented nuclei and prominent cytoplasmic granules typical of cultured mast cells as described (48). Duplicate aliquots of 10^6 cells were distributed to anti-IL-4-coated plates, activated with 1 μg/ml ionomycin and incubated undisturbed for 16 hrs. After washing, captured IL-4 was developed using the ELISPOT assay as described (46).

Cytokine analysis. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using real-time fluorogenic 5'-nuclease PCR (TaqMan™) was performed as described (2). Briefly, total cellular RNA was extracted, reverse transcribed using oligo$(dT)_{18}$ priming (Clontech Laboratories, Palo Alto, CA), and amplified using the primers as described (2). The specific signals for HPRT and IL-4 were at least 45-fold (5.5 cycles) over nonspecific background from RNA without prior reverse transcription.

Intracellular cytokine analysis was determined after activation with phorbol myristate acetate/ionomycin and brefeldin A blockade as described (2).

Parasites and infection. Infection with N. brasiliensis was performed as described (46). Briefly, third-stage larvae were isolated from feces of infected rats, washed extensively with PBS, counted and injected subcutaneously in PBS at the base of the tail using 750 worms per animal. After 8 days, animals were killed and the CD4+ T cells from the lungs and mesenteric lymph nodes isolated for cytokine detection as described (46). Serum was used to quantitate IgE by sandwich ELISA as described (46). The detection limit was 10 ng/ml.

Infection with L. major was performed as described (46). Briefly, animals were inoculated with 4×10^5 metacyclic promastigotes of L. major (strain WHOM/IR-/173) and the progression of disease monitored weekly using metric calipers to measure the size of the footpad lesions. After 6 weeks, serum was collected for IgE and the draining popliteal lymph nodes used to determine the numbers of IL-4-producing cells by ELISPOT assay. The numbers of viable parasites in the spleen were assayed using quantitative two-fold dilutions of tissue as described (46).

Experimental allergic airways disease. Anesthetized mice were sensitized 5 times by intranasal administration of 100 μg A. fumigatus antigen extract at 4 day intervals as described (49). After 4 days, airway hyperreactivity to escalating doses of acetylcholine was measured in anesthetized mice maintained on rodent ventilators in a whole-body plethysmograph as described (49). Airway reactivity is standardized to the PC_{200}, the

concentration of acetylcholine in μg/gm body weight resulting in a 200% increase over baseline, resting, total lung resistance. Lung CD4+ T cells were isolated over Ficoll and used to quantitate IL-4- and IL-5-expressing CD4+ T cells after stimulation with PMA/ionomycin and treatment with brefeldin A as described (19).

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Figure Legends

Figure 1. Generation of CNS-1 deficient mice

A. Strategy for targeted deletion of the CNS-1 element in ES cells. The genomic organization and a partial restriction map of the IL-4/IL-3 gene locus including the CNS-1 element (hatched block) is depicted at top as the 'wild-type locus'. The CNS-1 element was replaced by a loxP-flanked neomycin selection cassette (neo) as depicted in the 'targeting construct'. Integration of the targeting construct is depicted as the 'mutated locus'. Cre-mediated excision of the neo cassette in germline resulted in the final arrangement depicted as the 'mutated locus after cre recombination' at the bottom. Filled boxes: probes used for Southern blotting; triangles: loxP sites; H: HindIII; K: KpnI; N: NotI; tk: gancyclovir selectable expression cassette.

B. Southern analysis for correct integration of the targeting construct and germline transmission of the mutated allele. Genomic DNA was prepared from tail biopsies of mice, digested with HindIII, separated by electrophoreses and transferred onto membrane. The blot was hybridized with probe b. The wild-type locus results in an 8.8 kb fragment and the targeted locus in a 6.6 kb fragment. ch = chimera; +/- = heterozygous targeted; +/+ = wild-type.

C. Southern analysis for deletion of the neo cassette and the CNS-1 element. Genomic DNA was prepared from tail biopsies of mice, digested with KpnI, separated by electrophoreses and transferred onto membrane. The blot was hybridized with probe c. The wild-type locus results in a 1.8 kb fragment and the CNS-1 and neo-deleted locus in

a 1.3 kb fragment. +/+ = wild-type; +/- = heterozygous targeted; -/- = homozygous targeted.

Figure 2. Cytokine expression in vitro by CNS-1-deleted CD4+ T cells.

A. Naive CD4+ T cells were labeled with CFSE and stimulated under Th2 conditions. After 4 days, cells were re-stimulated with PMA/ionomycin and analyzed for intracellular IL-4. +/+ = wild-type cells; +/- = CNS-1-deleted heterozygous cells; -/- = CNS-1-deleted homozygous cells.

B. Cells from each division as assessed by CSFE dilution in panel A expressed as percent of cells in each division expressing IL-4 (left panel) and mean fluorescence intensity (MFI) of IL-4-positive cells as an index of IL-4 abundance (right panel). Black squares = wild-type; gray circles = heterozygous; open triangles = homozygous CNS-1 deleted.

C. Naive CD4+ T cells from wild-type (filled boxes) or CNS-1-deleted mice (open boxes) were activated for 5 days under Th2 conditions, washed extensively and re-stimulated with anti-TCR antibody. After 48 hr, supernatants were collected and analyzed for IL-4, IL-5 and IL-13 by ELISA.

Figure 3. IL-4 mRNA levels in CNS-1-deleted CD4+ T cells.

A. Highly purified naive CD4+ T cells from wild-type (filled squares), CNS-1 heterozygous (open circles) and CNS-1-deleted homozygous (open triangles) mice were stimulated using plate-bound anti-TCR/CD28 under Th2 conditions. RNA was purified at the designated times and used to template real-time fluorogenic RT-PCR assays to

quantitate amounts of IL-4 and HPRT transcripts. Data expressed as ratio of IL-4 to the constitutively-expressed HPRT transcript.

B. Naive CD4+ T cells from wild-type (filled boxes) or CNS-1-deleted (open boxes) mice were activated under Th2 conditions. After 5 days, RNA was purified and used to template a real-time RT-PCR assay for abundance of IL-4 and HPRT transcripts.

Figure 4. Numbers of IL-4-expressing mast cells is not altered by absence of CNS-1.

Left panel: Naive CD4+ T cells from wild-type (filled boxes) or CNS-1-deleted (open boxes) mice were activated under Th2 conditions, washed extensively after 5 days, and distributed to ELISPOT plates coated with anti-IL-4 antibody. After re-stimulation with PMA/ionomycin, the numbers of IL-4-secreting cells were determined after 16 hr of undisturbed incubation.

Right panel: Bone marrow-derived cultured mast cells from wild-type (filled boxes) or CNS-1-deleted (open boxes) were distributed to ELISPOT plates coated with anti-IL-4 antibody, stimulated with ionomycin and incubated undisturbed for 16 hr before determination of numbers of IL-4-expressing cells. Data from 1 of 2 comparable experiments.

Figure 5. Baseline serum IgE concentrations correlate inversely with the presence of CNS-1.

Sera from 10 age-matched, wild-type (filled boxes), CNS-1 heterozygous (hatched boxes) or CNS-1 homozygous (open boxes) deleted mice were assayed for IgE by ELISA. Data represent mean + SEM.

Figure 6. Impaired Th2 development in Nippostrongylus-infected CNS-1-deleted mice.

Cohorts of 3 wild-type (+/+) or CNS-1-deleted (-/-) mice were infected with N. brasiliensis. After 10 days, mice were killed and lymphocytes were isolated from the lung and mesenteric lymph nodes, activated using PMA/ionomycin, and CD4+ T cells were analyzed for the accumulation of intracellular IL-4 and IFN-γ. Percentages of positive cells indicated in respective gates. Lines delimit isotype antibody controls. Data from 1 of 2 comparable experiments.

Figure 7. Impaired Th2 development in CNS-1-deleted mice after Aspergillus-induced allergic airways disease.

A. Cohorts of 5 wild-type (+/+) and CNS-1-deleted (-/-) mice were sensitized repeatedly using Aspergillus antigens (ASP) or saline (PBS). Four days after the final challenge, mice were killed and lung CD4+ T cells purified, stimulated using PMA/ionomycin, and analyzed for intracellular accumulation of IL-4 and IFN-γ (left panels) and IL-5 and IFN-γ (right panels). Percentages of positive cells indicated in respective gates. Lines delimit isotype antibody controls.

B. Wild-type (filled bars) and CNS-1-deleted (open bars) mice were sensitized to Aspergillus antigen (ASP) or saline (PBS). Four days after the final challenge mice were anesthetized, maintained on rodent ventilators and total lung resistance to increasing doses of intravenously administered acetylcholine was measured using total body plethysmography. Data are depicted as the dose of acetylcholine in μg/gm body weight required to increase baseline lung resistance by 200% (PC_{200}).

C. Wild-type (filled bars) and CNS-1-deleted (open bars) mice were sensitized to Aspergillus antigens (ASP) or saline (PBS). Four days after the final challenge sera were collected and IgE assayed using ELISA.

Figure 8. Impaired Th2 development in CNS-1-deleted mice after L. major infection.

A. Cohorts of 10 wild-type (filled bars) and CNS-1-deleted (open bars) mice were infected with L. major promastigotes in the hind footpads. After 6 weeks, lesion size was measured using a metric caliper. Bars depict means, with individual mice depicted by circles.

B. Viable parasites recovered from spleens of 6 week-infected wild-type (filled bars) and CNS-1-deleted (open bars) mice. Individual mice depicted by circles.

C. Draining popliteal lymph nodes were collected from wild-type (filled bars) and CNS-1-deleted (open bars) mice 6 weeks after infection with L. major. Cells were activated using PMA/ionomycin and distributed over anti-IL-4-coated plates. After 16 hr, the numbers of IL-4-expressing cells was determined using ELISPOT assays.

D. Sera were collected from 6 week infected wild-type (filled bars) or CNS-1-deleted mice (open bars) and assayed for total IgE using ELISA.