



Identification of *Tapr* (an airway hyperreactivity regulatory locus) and the linked *Tim* gene family

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Published online: 28 November 2001, DOI: 10.1038/ni739

To simplify the analysis of asthma susceptibility genes located at human chromosome 5q23-35, we examined congenic mice that differed at the homologous chromosomal segment. We identified a Mendelian trait encoded by *T cell and Airway Phenotype Regulator (Tapr)*. *Tapr* is genetically distinct from known cytokine genes and controls the development of airway hyperreactivity and T cell production of interleukin 4 (IL-4) and IL-13. Positional cloning identified a gene family that encodes T cell membrane proteins (TIMs); major sequence variants of this gene family (*Tim*) completely cosegregated with *Tapr*. The human homolog of TIM-1 is the hepatitis A virus (HAV) receptor, which may explain the inverse relationship between HAV infection and the development of atopy.

Atopy, which includes asthma, allergic rhinitis and atopic dermatitis, is a complex trait that arises from environmentally induced immune responses in genetically susceptible individuals^{1,2}. The prevalence of all atopic diseases has greatly increased in industrialized countries over the past 20 years^{3,4}. Asthma, the most common chronic disease of childhood, now affects more than 15 million individuals in the United States and necessitates direct treatment costs that exceed US \$11 billion each year^{5,6}. Epidemiological studies suggest that the increase in asthma prevalence has resulted from improved hygiene and a reduction in the frequency of infections such as tuberculosis or hepatitis A within industrialized societies^{7,8}. However, the genetic polymorphisms that confer asthma susceptibility and the specific molecular pathways that might explain the increase in asthma prevalence are poorly understood.

The expression of asthma is influenced by multiple environmental and genetic factors that interact with each other in nonadditive ways, complicating the identification of asthma susceptibility genes. Asthma susceptibility has been linked to several chromosomal regions, but with a resolution no better than 5–10 cM, which typically includes hundreds of candidate genes. In addition, because the effects of genetic variation in any single gene are likely to have only modest effects in the overall pathogenesis of asthma and gene-gene and gene-environment interactions confound the analysis, the localization of regions amenable to positional cloning has proven difficult. Nevertheless, asthma susceptibility has been linked to chromosomes 5, 6, 11, 14 and 12^{9–15}. Of these, chromosome 5q23-35 has received the most attention^{9,10,16–20} because it contains a large number of candidate genes, including interleukin 9 (IL-9), IL-12p40, the β -adrenergic receptor and the IL-4 cytokine cluster, which contains the genes that encode IL-4, IL-5 and IL-13. However, the large size of the linked region of 5q has complicated its analysis, and an asthma susceptibility gene from

this site has not been conclusively identified.

To analyze the human 5q23-35 region for asthma susceptibility genes, we used a mouse model that offers several potential advantages. Environmental variation can be controlled, multiple phenotypes can be tested simultaneously and inbred strains can be sensitized with allergen to develop airway hyperreactivity (AHR), a hallmark of human asthma^{21,22}. In addition, we used congenic inbred mouse strains that differed only by a small chromosomal region homologous to human chromosome 5q, thereby allowing us to study this region in the absence of genetic variation outside the region.

We used this strategy to delineate a single chromosomal region that confers reduced T helper type 2 (T_H2) responsiveness and protects against AHR. Thus, we define here *T cell and airway phenotype regulator (Tapr)*, a locus that is genetically separable from the IL-4 cytokine gene cluster and other nearby cytokine genes, such as that encoding IL-12p40. Within the region associated with *Tapr*, we positionally cloned a gene family that encodes T cell membrane glycoproteins with conserved immunoglobulin variable domain (IgV) and mucin domains. Because of their predicted structure, we termed the protein products of these family members, which are expressed on T cells, as T cell, immunoglobulin domain and mucin domain (TIM) proteins. We identified major polymorphisms in TIM-1 and TIM-3 that were strongly associated with T_H1 - T_H2 differentiation and the expression of AHR in congenic mice. The human homolog of TIM-1 is the cellular receptor for hepatitis A virus, HAVcr-1. Our findings therefore suggest that CD4⁺ T cells and TIM-1 may mediate the protective effect of previous infection with hepatitis A on the development of atopy. Because the prevalence of hepatitis A infection is greatly reduced in industrialized countries, our findings may explain in part the large increase in asthma prevalence in these countries over the past 20 years.

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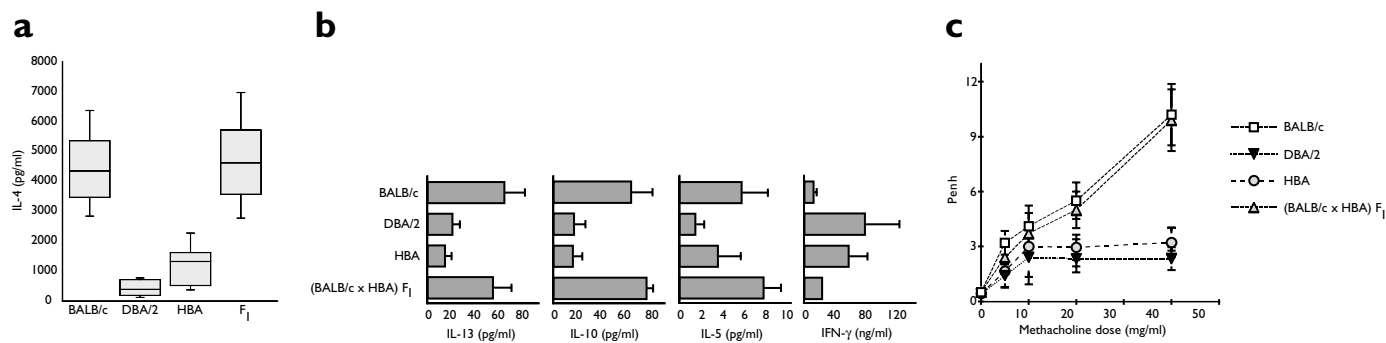


Figure 1. HBA cytokine and AHR responses resemble DBA/2 phenotypes. (a) The concentrations of IL-4 produced by BALB/c and by (BALB/c×HBA)_{F1} cells were significantly higher than those produced by HBA cells ($P<0.0001$). Lymph node cells from mice immunized with KLH were collected, B cell-depleted and cultured in cDME (complete Dulbecco's Modified Eagle's Medium) with KLH. Supernatants were collected after 96 h and IL-4 production measured by ELISA. A box and whisker plot⁶² of IL-4 concentrations is shown ($n=10$ in each group). The bars represent the full range of data. The boxes encompass the second and third quartiles and the median of the dataset is the bar inside each box. (b) HBA mice produced significantly less IL-13 and IL-10 than BALB/c or (BALB/c × HBA)_{F1} mice ($P<0.0001$). Data are the mean±s.d. cytokine concentrations produced by lymph node cell cultures; $n=10$ mice per group. (c) Allergen-induced airway hyperreactivity was significantly greater in BALB/c than HBA mice ($P<0.0001$, with 40 mg/ml methacholine). Pulmonary airflow obstruction was measured in mice that were challenged with methacholine. (BALB/c × HBA)_{F1} showed BALB/c phenotypes. Data are mean±s.e.m. Penh values. In all experiments, Student's *t*-tests were used to assess significance.

Results

IL-4 production and AHR in HBA mice

BALB/c mice develop T_H2 -biased immune responses with enhanced AHR, whereas DBA/2 mice develop reduced IL-4 responses²⁶ that protect them against the development of AHR^{27–29}. We examined congenic mice produced on a BALB/c genomic background with discrete genomic intervals inherited from individual DBA/2 chromosomes^{23–25}. The congenic mice are not fundamentally different from any other inbred strain, but simplify the genetic analysis because they allow the characterization of a single locus without interference from multiple epistatic genes that also influence the asthmatic phenotype. By screening several of these congenic strains for reduced T_H2 responsiveness, we identified one congenic line, C.D2 Es-Hba (HBA), which contained a segment of chromosome 11 inherited from DBA/2 mice that was homologous to human 5q23–35. As expected, lymph node cells from immunized control BALB/c mice produced high concentrations of IL-4, which confirmed the tendency of BALB/c mice to develop T_H2 -biased immune responses (Fig. 1a)²⁶. In contrast, lymph node cells from HBA mice produced significantly lower concentrations of IL-4 ($P<0.0001$), which were similar to those observed in DBA/2 mice. Compared to BALB/c mice, HBA mice also produced significantly less IL-13 ($P<0.0001$) and IL-10 ($P<0.0001$) as well as lower amounts of IL-5, whereas production of IFN- γ was increased (Fig. 1b). These results showed that the DBA/2-derived region of HBA chromosome 11, which has large regions of conserved synteny with human 5q23–35, contains a gene that reduced antigen-specific IL-4, IL-13 and IL-10 production, enhanced IFN- γ production and converted the BALB/c cytokine phenotype into a DBA/2 cytokine phenotype.

The capacity of HBA mice to develop antigen-induced AHR, which is associated with T_H2 -biased immune responses, was examined^{27–29}. Upon sensitization and challenge with allergen, control BALB/c mice developed high AHR, whereas similarly immunized HBA congenic mice, like DBA/2 mice, expressed normal airway reactivity in response to methacholine (Fig. 1c). Together these results strongly suggested that genetic variation in a single locus on chromosome 11 regulated both T_H2 cytokine production and AHR; therefore, we tentatively designate the relevant genetic determinant(s) in HBA mice as a single locus, *T cell and Airway Phenotype Regulator (Tapr)*.

We also examined (BALB/c × HBA)_{F1} mice, which, like the BALB/c mice, produced high concentrations of IL-4, IL-13 and IL-10 (Fig. 1a,b)

and developed elevated antigen-induced AHR (Fig. 1c). The BALB/c congenic HBA mice were homozygous for the relevant DBA/2-derived congenic regions, whereas the (BALB/c×HBA)_{F1} mice are heterozygous for this region. Thus, these results showed that a DBA/2 allele on chromosome 11, in isolation from other genes that regulate IL-4 synthesis, reduced IL-4 production and AHR in a recessive manner.

Genetic mapping of *Tapr*

The congenic region in HBA mice was delineated with 36 genome-wide markers²³, which included two chromosome 11 markers and the hemoglobin- $\alpha 2$ (*Hba-a2*) and esterase 3 (*Es3*) loci. The HBA genome, outside of chromosome 11, was inherited from BALB/c mice. A more precise analysis, which used 25 simple sequence length polymorphism (SSLP) markers that are polymorphic between DBA/2 and BALB/c mice³⁰, showed that HBA mice inherited two segments of chromosome 11 from

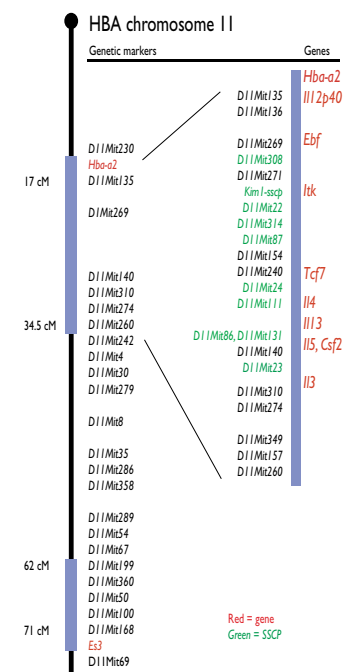


Figure 2. Regions of HBA chromosome 11 were inherited from DBA/2. The HBA chromosome 11 contains two regions derived from DBA between *Hba-a2* and *Es3*, as delineated by SSLP markers. (Left) The regions of HBA chromosome 11 with DBA/2 genotypes are highlighted (in blue). The markers provided a 2–5-cM resolution distal to *D11Mit230*. (Right) Where the mouse chromosome 11 had regions of highly conserved synteny with 5q23–35, additional markers were identified with informative polymorphisms between BALB/c and DBA/2, to provide 0.01–2-cM resolution in this region. Single-stranded conformation polymorphisms (SSCP) markers are in green to distinguish them from the SSLP markers; the positions of particular genes of interest (in red) are also shown. Where the arrangement of the marker map differed from the Chromosome Committee Reports and the MIT linkage map, our map concurred with previous linkage³⁵ and physical maps^{19,63,64}.

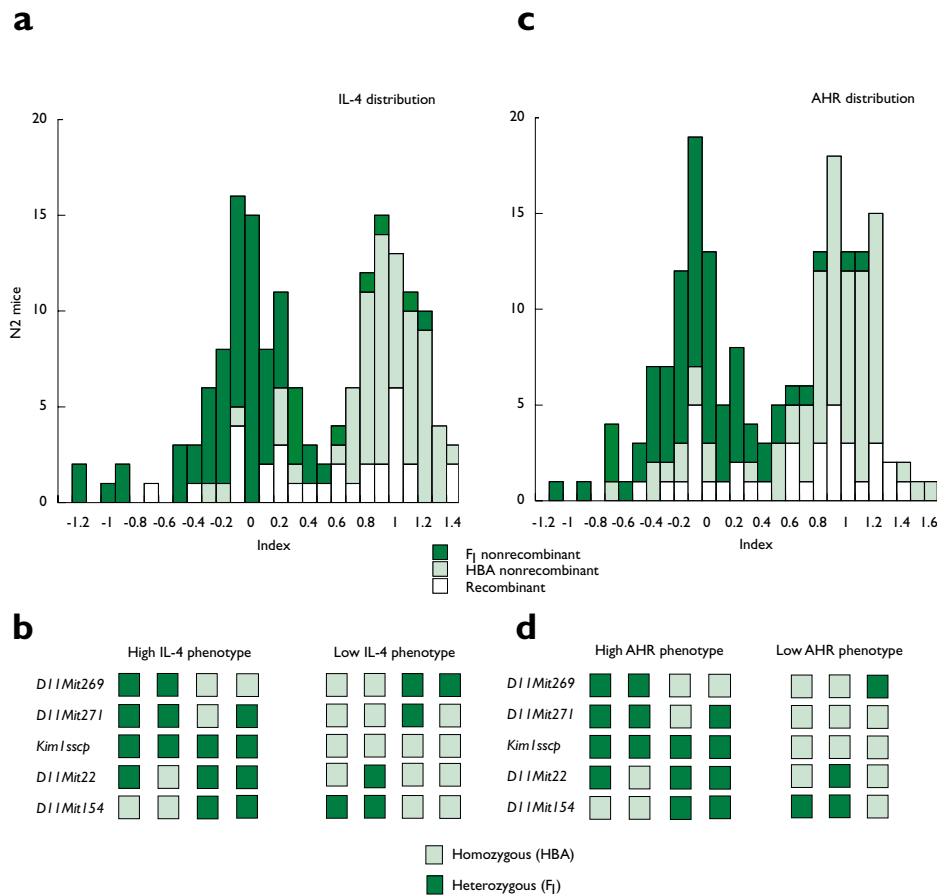


Figure 3. *Tapr* cosegregates with *Kim1sscp* (a) IL-4 production by N2 mice is bimodal, with peaks corresponding to F₁ and HBA phenotypes. To evaluate the relative phenotypes of recombinant N2 mice in multiple experiments, we used an indexing function that allowed us to consolidate data from multiple experiments (see Methods). The histogram shows a bimodal distribution of IL-4 index values for N2 mice with (BALB/c × HBA)F₁, HBA homozygous and recombinant haplotypes shown. Distributions associated with the F₁ and HBA haplotypes were distinct ($P < 0.0001$). (b) IL-4 regulation cosegregated with *Kim1sscp*. Recombinant N2 haplotypes were sorted by IL-4 index values into groups associated with high IL-4 phenotypes (index < 0.35) and low IL-4 phenotypes (index > 0.65). Each column of boxes represents a recombinant haplotype. Alleles for these haplotypes at loci between *D11Mit269* to *D11Mit154* are shaded according to genotype. The IL-4 phenotype was linked to *Kim1sscp* in all haplotypes examined. (c) AHR of N2 mice is bimodal, with peaks that corresponded to F₁ and HBA phenotypes. Index values calculated from Penh values are shown. The histogram shows a bimodal distribution of AHR index values for N2 mice. (BALB/c × HBA)F₁, HBA homozygous and recombinant haplotypes are indicated. Distributions associated with the F₁ and HBA haplotypes were distinct ($P < 0.0001$). (d) AHR regulation cosegregated with the IL-4 regulation and *Kim1sscp*. Recombinant N2 haplotypes were sorted by AHR index values into groups associated with high and low AHR phenotypes, as in a. Each column of boxes represents a recombinant haplotype. Alleles for these haplotypes at loci between *D11Mit269* to *D11Mit154* are shaded according to genotype. The AHR regulatory locus was linked to *Kim1sscp*, between *D11Mit271* and *D11Mit22*, in all haplotypes examined. In all experiments, Student's *t*-tests were used to assess significance.

DBA/2 mice (Fig. 2, left column). The proximal region contained a 20-cM segment with homology to human chromosome 5q23-35, which afforded the possibility that a genetic locus implicated in human asthma linkage studies could be identified in a mouse model of asthma.

To map the T_H2-AHR-controlling locus *Tapr* at higher resolution (BALB/c × HBA)F₁ mice were backcrossed to HBA mice to generate N2 animals. With this backcross approach, the set of alleles contributed by the HBA parent is predetermined, and the set of alleles contributed by the F₁ parent can be assessed by genotyping³¹. Thus, recombination events that produce informative haplotypes within the congenic region can be detected in the N2 mice and used to assess the linkage of *Tapr* to loci in the congenic interval. Because of the recessive nature of *Tapr*, we tested N2 mice from these backcrosses to identify the minimum homozygous region of HBA-derived genes that was sufficient to confer the HBA *Tapr* phenotype. More than 2000 N2 animals were generated and genotyped. Using SSLP markers, we selected those N2 mice with informative recombination events and phenotyped them according to their capacity to produce IL-4 in response to immunization with keyhole limpet hemocyanin (KLH). In this primary analysis, we determined that the relevant locus resided within the proximal congenic region, between *D11Mit135* and *D11Mit260*. To map *Tapr* at higher resolution, 22 additional markers were identified and used (Fig. 2, right column) to provide 0.1–1-cM resolution in the area of interest.

To accurately compare the results of IL-4 cytokine analyses done over several months, we generated an IL-4 index for each N2 mouse used in the experiments (see Methods). We found that the index values fell within a

bimodal distribution (Fig. 3a), in which the phenotype index associated with N2 mice that had nonrecombinant HBA genotypes was significantly higher ($P < 0.0001$, with a paired Student's *t*-test) than the phenotype index associated with N2 mice that had nonrecombinant (BALB/c × HBA)F₁ genotypes. For the mice with unique genotypes, we used several methods to ensure that the single measurements of cytokine production and AHR were adequate, as this was critical in linkage analysis. First, simultaneous to testing each of the N2 mice with the recombinations of interest, we also tested the nonrecombinant siblings, which had either an HBA or (BALB/c × HBA)F₁ genotype. We generated N3 mice by crossing selected N2 mice with recombinations of interest back to HBA in order to produce additional individual mice with that particular genotype. All data were the means of data pooled from the individual mice tested with a given genotype. Therefore, we were confident of our measures of cytokine production and AHR and that we had overcome assay variations that were due to variables inherent in biological systems.

Because the IL-4 values associated with the N2 mice that inherited recombinant haplotypes segregated in a bimodal distribution (Fig. 3a), we were able to show that the genetic locus that controls high IL-4 responses is located between markers *D11Mit271* and *D11Mit22* (Fig. 3b). In addition, we observed high IL-4 production in mice with a BALB/c allele present at *Kim1sscp*, and low IL-4 production in mice with homozygous HBA genotypes at *Kim1sscp*. Thus, *Tapr* was nonrecombinant with *Kim1sscp*, an intronic marker within a mouse homolog of *Rattus norvegicus* kidney injury molecule (*Kim1*) (D. Beier, personal communication)^{32,33}. In contrast, *Tapr* segregated from all other markers with at least

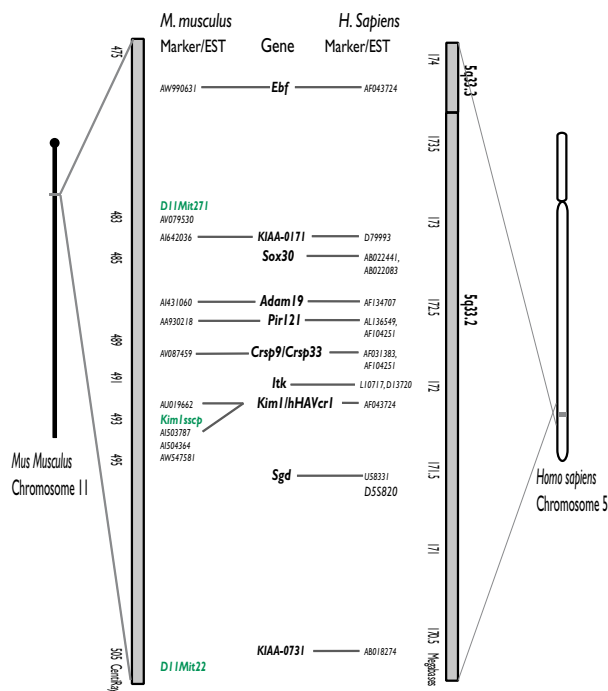


Figure 4. Mouse chromosome 11 interval containing *Tapr* is highly homologous to human 5q33. To construct a composite map around the *Tapr* locus, we integrated available information from the mouse linkage, backcross and radiation hybrid maps and identified a region of highly conserved synteny in current maps of the human genome. ESTs located on a physical map^{30,36} of mouse chromosome 11 are denoted by their accession numbers and aligned by homology to genes, which correspond to ESTs on human chromosome 5^{37,38}. All known genes between *KIAA0171* and *Sgd* are shown. Markers included in the maps of Fig. 3 are shown here in green.

one recombination. The fact that *Tapr* and *Kim1sscp* segregated together showed that the *Tapr* locus is located very close to, or is indistinguishable from, *Kim1sscp*. Based on the frequency of recombinant haplotypes between *D11Mit271* and *D11Mit22*, we calculated a recombination frequency of 0.0039, which indicated that the *Tapr* locus maps to a small, 0.3–0.5-cM, region. We also calculated a recombination frequency of 0.08 for *Tapr* and the gene encoding IL-4. Therefore, *Tapr* is located 5–10 cM away from the IL-4 cytokine cluster but is within a region of the mouse genome that has been linked to autoimmune disease susceptibility³⁴ and is homologous to regions of human chromosome 5q that are linked to human atopy and asthma.

Using an analogous approach, we examined the segregation of allergen-induced AHR phenotypes in mice with informative recombinant haplotypes. With indexed AHR values, N2 mice showed parental phenotypes, which produced a bimodal distribution in a histogram of AHR index values in a group of sensitized N2 mice (Fig. 3c). By analyzing the segregation of AHR phenotypes associated with >1000 N2 mice, we showed that the genetic locus that controls AHR responses is also located between markers *D11Mit271* and *D11Mit22* (Fig. 3d) and that the AHR phenotype was nonrecombinant with *Kim1sscp*. Thus, we showed that both IL-4 responsiveness and AHR cosegregate with the *Tapr* locus, which suggested that the same locus regulates both IL-4 expression and AHR (Fig. 3). These findings also show that the *Tapr* locus is >5 cM centromeric to the IL-4 cytokine cluster and the cytokine genes in the cluster, previously thought to be “candidate” atopy or asthma susceptibility genes. Our mapping results also establish that *Tapr* is genetically separable from both the gene encoding IL-12p40 and the region of mouse chromosome 11 that includes the T_H1 -IL-12 regulatory locus *Tpm*³⁵.

Physical mapping of *Tapr* to human 5q33

To construct a composite map around the *Tapr* locus, we integrated available information from the Mouse Genome Database linkage, backcross and radiation hybrid maps and identified a region of conserved synteny in maps of the human genome. Radiation hybrid maps place genetic markers near *D11Mit271* and *D11Mit22*, including several expressed sequence

tags (ESTs) with homology to known genes or unigene clusters, onto a physical map of the mouse genome^{30,36}. We further examined these markers and their associated ESTs for similarity to known gene clusters. We assembled these markers onto a scaffold for comparison to the human genome. Using this approach, we found marked similarity between specific mouse radiation hybrid markers and the following human genes: *KIAA0171*, *Adam19*, *Sox30*, *Pir121*, *Crsp9* (*Crsp33*) and *hHAVcr1*. Once we had placed these genes on a physical map of the mouse genome between our flanking markers (Fig. 4), we were able to locate those genes in the Human Genome Browser (version 3, University of California at Santa Cruz, March 2001)^{37,38}.

The high degree of conservation between the mouse and human genomes in this region indicated linkage of the *Tapr* locus to human 5q33.2. We identified all known genes and ESTs in this region of the human map (Fig. 4)^{37–39}. Known genes of immunological interest near *Kim1sscp* included IL-2-inducible T cell kinase (*Itk*) and a coregulator of the SP-1 transcription factor (*Crsp9*), which are both involved in T cell differentiation^{40,41}. We sequenced coding regions from both of these candidate genes and found no coding polymorphisms in either *Itk* or *Crsp9*.

Identification of the *Tim* gene family

Because the mouse homolog of rat *Kim1* is located within the 0.4-cM region and is tightly linked with *Tapr*, we identified clusters of ESTs with similarity to rat *Kim1*. These ESTs provided only partial coverage and contained large segments of variation. The closest human homolog of *Kim1* is the human hepatitis A virus cellular receptor *hHAVcr1*. Sequence similarity searches of the human and mouse genomes suggested that two or more additional homologs of *Kim1*, perhaps members of the same gene family, are also located on human chromosome 5 and mouse chromosome 11.

Using cDNA from concanavalin A (Con A)-stimulated splenocytes, we identified and cloned two mouse homologs of *Kim1*, which we designated *Tim1* and *Tim2*, that mapped to the *Tapr* region (Fig. 5a). TIM-3—a third, more distantly related, TIM family member—has also been cloned and identified as a surface protein on differentiated T_H1 cells⁴².

All three members of this gene family are expressed by stimulated T cells, and all three forms map to the *Tapr* region of mouse chromosome 11 where they encode cell-surface glycoproteins with common structural motifs, including a signal peptide, Ig domain, mucin domain, transmembrane region and intracellular tail with phosphorylation sites. Therefore, we designated the genes as belonging to a *T cell, immunoglobulin domain, mucin domain* (*Tim*) gene family. Mouse *Tim1* is the ortholog of *Kim1* in rats and of *HAVcr1* in humans and African green monkeys. *Tim2* has no known orthologs.

Mouse *Tim1* encodes a 305-amino acid (aa) membrane protein that has 78% identity with rat KIM-1 and 42% identity with human HAVcr-1. The multiple sequence alignment with mouse TIM-1, rat KIM-1, human HAVcr-1 and African green monkey HAVcr-1 showed the degree of homology between the TIM-1, KIM-1 and HAVcr-1 proteins in these species (Fig. 5b). The cytoplasmic region of TIM-1 contains two tyrosine residues and includes a highly conserved tyrosine kinase phosphorylation

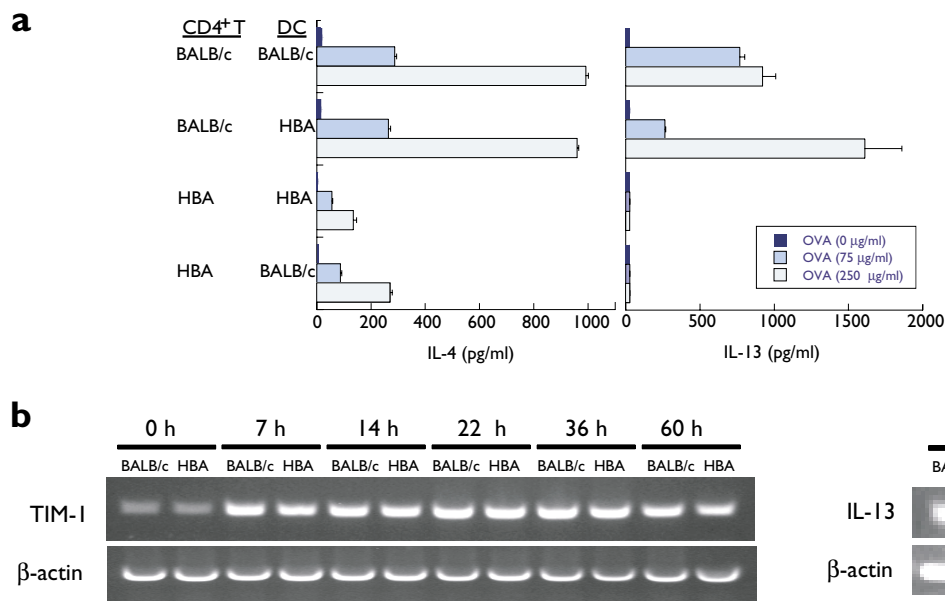


Figure 6. *Tapr* regulates CD4⁺ T cell IL-4 and IL-13 responses. (a) T cells from BALB/c DO11.10 mice produced higher amounts of IL-4 and IL-13 in response to antigen than T cells from HBA DO11.10 mice. Splenic CD4⁺ T cells were isolated by positive selection with anti-CD4 magnetic beads, then cultured with bone marrow-derived DCs and OVA. After 7 days, the cells were restimulated. Supernatants were collected after 18–24 h of secondary culture. Data are mean±s.d. cytokine concentrations. (b) Detection of expression of TIM-1 mRNA by RT-PCR of total RNA from purified CD4⁺ T cells. CD4⁺ T cells were isolated from primary cultures, then cultured with bone marrow-derived DCs and OVA. TIM-1 expression was detected in both BALB/c DO11.10 and HBA DO11.10 CD4⁺ T cells throughout the primary culture. As expected, by 36 h of primary culture, IL-13 mRNA levels were higher in BALB/c DO11.10 than in HBA DO11.10 cells.

polymorphisms, not splicing variants. By sequencing further genomic segments of TIM-1 and TIM-3 in other mouse strains, we found that C57/BL6, a strain that is similar to DBA/2 with respect to its tendency to develop reduced T_H2 and AHR responses, also had the HBA(DBA/2) *Tim1* and *Tim3* alleles (data not shown). The polymorphisms in TIM-1 were located in the signal and mucin-like domains, whereas the polymorphisms identified in TIM-3 were clustered in the Ig domain (Fig. 5c). In glycoproteins with Ig and mucin domains, variants in either domain may affect receptor-ligand interactions, as shown for MAdCAM-1. The MAdCAM-1 Ig domains are critical for binding the integrin $\alpha_4\beta_7$, and the glycosylated mucin domain is required for binding to CD62L. In addition, although the predicted cleavage site of TIM-1 was unaltered by polymorphism in the signal sequence, it is possible that the polymorphism may have affected the efficiency of cleavage and/or trafficking of the receptor to the cell surface. These mouse *Tim* sequences and polymorphisms will be important for future research relating not only to immune responses, but also to HAV viral pathogenesis in humans.

Analysis of genomic DNA samples from our N2 backcross (Fig. 3) showed that the TIM-1 and TIM-3 polymorphisms cosegregated completely with *Tapr*. Although these observations did not determine the extent to which changes in TIM-1, TIM-3 or both were responsible for changes in AHR and T_H2-mediated inflammation, we suggest that polymorphisms in human TIM-1 (hHAVcr-1) and/or TIM-3 underlie the strong association between asthma susceptibility and human chromosome 5q. This idea was supported by the fact that major variants in coding regions of human *Tim1* were evident on examination of the human genome and EST databases. Comparison of these human cDNA variants with variants of monkey HAVcr-1⁴⁴ and the mouse variants identified here showed that there was extensive variation in the predicted protein sequences of TIM-1 (Fig. 5b,c). This high degree of variation distinguished TIM-1 and its family members from many other candidate genes, such as the cytokines and the cytokine receptors that have been most closely studied as asthma susceptibility candidate genes. In addition, the association between *Tim1* and asthma susceptibility was further supported by reports of significant linkage of mite-sensitive childhood asthma to D5S820 (mean LOD score = 4.8), a marker which is ~0.5 megabases from *Tim1* and *Tim3* (Fig. 4)¹⁷.

T cells confer the *Tapr* effect

To better understand the function of the *Tapr* locus, we determined whether allelic variation of *Tapr* affected the function of T cells or of antigen-presenting cells (APCs). For these experiments, we generated ovalbumin (OVA)-specific TCR-transgenic mice (Tg) on the HBA background (HBA DO11.10 mice). We compared the *in vitro* cytokine profiles of CD4⁺ T cells purified from HBA DO11.10 mice with those of analogous TCR-Tg CD4⁺ T cells derived from mice with a BALB/c background (BALB/c DO11.10). We cultured purified CD4⁺ T cells from each of these strains with OVA and dendritic cells (DCs) derived from either BALB/c or HBA bone marrow, and evaluated the cytokines produced.

BALB/c DO11.10 T cells produced higher amounts of IL-4 and IL-13 than the HBA DO11.10 T cells did, in a manner that was independent of the APC source (Fig. 6a). The source of the CD4⁺ T cells, however, did determine the amount of IL-4 or IL-13 produced at each antigen concentration, regardless of the APC source during either the primary or secondary stimulation. We detected equivalent concentrations of IL-12 in culture supernatants for each combination of cell types (data not shown), which also showed that BALB/c and HBA DC function were comparable. In addition, BALB/c DO11.10 and HBA DO11.10 T cells produced equivalent amounts of IL-2 and showed comparable amounts of proliferation in response to OVA during the secondary cultures (data not shown). These results showed that, although HBA and BALB/c T cells were similarly activated, the amounts of T_H2 cytokines they produced were distinct.

During the primary culture of DO11.10 T cells with DCs and OVA, TIM-1 mRNA was expressed by both BALB/c and HBA CD4⁺ T cells (Fig. 6b). After 36 h of primary stimulation, BALB/c DO11.10 T cells produced more IL-13 mRNA than HBA DO11.10 T cells (Fig. 6). Supernatants collected after 4 days showed similar results when IL-13 was assessed by ELISA (data not shown). Thus, during the primary response to antigen, BALB/c CD4⁺ T cells developed a stronger T_H2 response than HBA CD4⁺ T cells. This showed that *Tapr* regulates T_H cell differentiation during primary antigen-specific responses, when early cytokine expression is critical in determining the subsequent T_H phenotype^{45,46}. The expression of TIM-1 during the earliest stages of these responses suggested that the BALB/c *Tim1* allele promotes early expression of T_H2 cytokines, whereas the HBA allele is associated with down-regulation of T_H2 cytokines.



Discussion

We have mapped *Tapr*, a locus that regulates the development of T_H2 cytokine production and antigen-induced AHR in a mouse model that mimics the pathophysiology of human asthma. We localized *Tapr* using an interval-specific congenic mouse (HBA) that carried a chromosomal segment with homology to human chromosome 5q, a region of the human genome that has been repeatedly linked to atopy and asthma. Using this congenic mouse strategy we converted a complex trait into a simpler, possibly single-gene, trait. This allowed us to avoid the major difficulties usually associated with identification of susceptibility genes of complex traits, which result from the interactions of multiple independently segregating genes that, together, overwhelm the effects of any given gene. With this congenic mouse strategy, we narrowed the interval of *Tapr* to 0.4 cM, sequenced several candidate genes in this region and positionally cloned the *Tim* family of genes.

We identified and cloned the full cDNA sequence for TIM and identified marked polymorphisms in the TIM-1 proteins of BALB/c compared to HBA mice. We found that the BALB/c sequences for TIM-1 and TIM-3 were associated with susceptibility to AHR and allergic T cell responses, whereas the HBA sequences were associated with protection against these responses. TIM-3 is preferentially expressed by differentiated T_H1 cells⁴². The association of polymorphic *Tim3* variants with *Tapr* suggested that TIM-3 might regulate T_H1 and T_H2 cell function. However, the variations in *Tim3* might also be attributed to a haplotype tightly linked to *Tim1*.

We believe that TIM-1 plays an important role in the regulation of asthma and allergic disease for several reasons. First, *Tim1* is expressed in CD4⁺ T cells, which play a critical role in the development of AHR and in the pathogenesis of asthma²⁹. Second, *Tim1* is transcribed during primary antigen stimulation, a period of time that is crucial in influencing T cell differentiation and commitment to T_H2 cytokine production and the development of AHR. Third, TIM-1 is likely important in regulating asthma because the human homolog of TIM-1 serves as a receptor for HAV. We propose that, *via* its interaction with TIM-1, HAV may reduce T_H2 differentiation and thereby reduce the likelihood of developing asthma. Such an interaction between HAV and TIM-1 would explain the inverse association of HAV infection with the development of asthma and allergy^{8,47,48}. This hypothesis is supported by other examples of viral interactions with viral receptors that regulate T_H cell differentiation. For example, by binding to SLAM on CD4⁺ T cells, the measles virus inhibits T_H1 differentiation by interfering with the natural pathway by which SLAM regulates T_H1 - T_H2 differentiation^{49,50}. Asthma susceptibility alleles of *Tim1* may have been preserved through human evolution because they provide a protective effect, such as resistance to fulminant HAV-induced hepatitis or resistance to autoimmune disease. This hypothesis is supported by the fact that the HAV receptor in primates is highly variable⁴⁴ and mutations in the genes encoding cell surface molecules that serve as viral receptors often alter susceptibility to infection⁵¹. For example, the chemokine receptor mutation, Δ CCR5, provides resistance to HIV infection⁵². Finally, the remarkable amount of *Tim1* sequence polymorphism that occurs in the coding region of mouse *Tim* alleles, as well as in monkey *Tim1* and human *TIM1* (observed in the human genome and EST databases), suggested that *Tim1* is a major asthma susceptibility gene in the human chromosome 5q23-35 region. In other studies of this region, most attention focused on candidate genes within the cytokine cluster and conclusive identification of an asthma susceptibility gene in this region was not possible, as only minor sequence polymorphisms were found in the coding regions of these genes. In contrast, sequence variation in *Tim1* is marked and reminiscent of other gene families that play central roles in immunology⁵³. Validation of the role of TIM-1 in asthma awaits the sequencing of TIM-1 in humans

and association analysis of genetic variations in *TIM1* in subjects with asthma and allergy.

In summary, we used congenic mice to locate a strong candidate asthma susceptibility gene and overcome inherent difficulties in the examination of this complex genetic trait. We identified a gene family that exists in a region homologous to human chromosome 5q, and which may play a major role in T_H cell development and in asthma susceptibility. Although previous studies in humans identified several candidate genes on human chromosome 5q, the *Tim1* gene product we identified also provided an explanation for the inverse relationship between HAV infection and reduced asthma susceptibility. Further analysis of this gene family is required to consolidate our understanding of these genes.

Methods

Animals. Congenic lines, including HBA, were generated by introgressively backcrossing DBA/2N mice onto a BALB/cAnPt background. BALB/cBy, DBA/2J and (BALB/c×DBA/2) F₁ mice (CByD2F1/J) were from the Jackson Laboratory (Bar Harbor, ME), whereas BALB/cAn and DBA/2N were from Taconic Labs (Germantown, NY). (BALB/c × HBA)F₁ mice were generated by crossing BALB/cByJ and HBA mice. A total of 2,147 N2 mice were generated by backcrossing (BALB/c×HBA)F₁ to HBA mice. In our analysis of recombinant N2 animals, recombinant mice were tested along with nonrecombinant siblings whenever possible. To examine individual N2 genotypes in multiple assays, we preserved selected recombinant haplotypes by backcrossing selected N2 to HBA mice to generate N3 mice. These mice were then genotyped to select mice with the recombinant N2 haplotype. DO11.10 mice, which are transgenic for the TCR that recognizes OVA(323–339) and backcrossed to BALB/c⁵⁴, were from D. Loh and were bred at our facilities. HBA DO11.10 mice were produced by backcrossing DO11.10 to HBA mice. DO11.10 mice were selected, by fluorescence-activated cell sorting (FACS) analysis, for the transgenic TCR and genotyped to select for HBA alleles between *D11Mit135* and *D11Mit168*. The Stanford University Committee on Animal Welfare approved all animal protocols.

Genotyping. Additional markers around the *Tapr* locus were identified by testing all available *D11Mit* markers present between *D11Mit140* and *D11Mit269* and all radiation hybrid markers near *D11Mit271* and *D11Mit22* for any polymorphisms between DBA/2 and BALB/c⁵⁰. MIT MapPair primers were from Research Genetics (Huntsville, AL), all other primers were synthesized in the Protein and Nucleic Acid Facility (Stanford, CA). Primers for *Kim1sscp* were: 5'-CTAGTACTCAGTTGAAGTCCAGAAAGGTGAG-3' and 5'-GAGATCAGAAAACCAGTACTGG-3'. Polymerase chain reaction (PCR) analysis was done according to Research Genetics protocols, and SSCP polymorphisms were resolved with 4–5% Metaphor agarose (BioWhittaker, Walkersville, MD). Products analyzed for SSCP were amplified with [³²P]dCTP and separated on denaturing acrylamide gels, at 40 W and 4 °C, with a Sequi-Gen GT System (Bio-Rad, Hercules, CA).

Immunization protocols. Mice studied in cytokine production assays were primed with KLH (Calbiochem, La Jolla, CA) in complete Freund's adjuvant (CFA). For measurement of AHR, mice were immunized intraperitoneally with 50 µg of OVA complexed with aluminum potassium sulfate (alum) on day 0 and intranasally with 50 µg of OVA in 50 µl of PBS after light anesthesia on days 7, 8 and 9. Control mice received intraperitoneal injections of alum alone and intranasal PBS. AHR to inhaled methacholine was measured 24 h after the last intranasal dose of OVA (day 10).

Measurement of airway responsiveness. Airway responses were assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole-body plethysmograph (Buxco Electronics Inc., Troy, NY), as described^{27,55}. The peak enhanced pause (Penh) measurement has been studied intensively by many groups and gives an equivalent measure of AHR as do more invasive "physiological techniques"^{56,57}.

Cell culture. Lymph node cells from mice primed with KLH were prepared as described⁵⁸. Transgenic DO11.10 CD4⁺ T cells were positively selected using MACS columns after incubation with anti-CD4 magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells (2×10⁶ cells/well) were cultured in 96-well round-bottomed plates with 250 µg/ml of OVA and 1×10⁶ bone marrow-derived DCs/well. After 7 days, the DO11.10 T cells were washed and restimulated with fresh APCs and antigen. Antigen concentration for the primary DO11.10 cultures was titrated during the restimulation, as in Fig. 6. Bone marrow-derived DCs were generated as described, but with some modifications: 5×10⁶ bone marrow cells were cultured in 9-cm diameter tissue culture dishes with 10 ml of culture medium that contained 20–25 U/ml granulocyte-macrophage colony-stimulating factor⁵⁹. Loosely adherent cells were transferred onto a second dish on the day 6 of culture; within 4 days, these transferred cells were used as a source of DCs.

Cytokine ELISA and monoclonal antibodies. ELISAs were done as described^{58,60,61}. Monoclonal antibodies for ELISA and FACS analysis were purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography. IL-13 antibodies were from PharMingen (San Diego, CA). Anti-clonotypic KJ1-26.1 was from P. Marrack, National



Jewish Medical Center; it was conjugated to fluorescein isothiocyanate according to standard protocols before FACS.

RNA analysis, cloning, and sequencing. Total RNA from Con A-stimulated splenocytes was reverse transcribed with Gibco Superscript II (Gibco-BRL, Gaithersburg, MD). PCR products of full-length TIM-3 cDNA were amplified, purified with Qiagen QIAquick PCR purification reagents (Qiagen, Valencia, CA), and sequenced directly by Biotech Core (Mountain View, CA). PCR products for TIM-1 and TIM-2 cDNA were cloned into electrocompetent TOP10 cells with TOPO-XL cloning reagents (Invitrogen, Carlsbad, CA). Plasmids were purified with a standard alkaline lysis protocol. BALB/c and HBA plasmids were sequenced by Biotech Core (Mountain View, CA).

Calculation of IL-4 indexes. To accurately compare the results of IL-4 cytokine analyses done over several months, an IL-4 index for each experiment was generated for each N2 mouse using the calculation $(B - x)/(B - H)$, where B = IL-4 production by cells from BALB/c mice, H = IL-4 production by cells from HBA mice and x = IL-4 production by cells from the N2 mouse being assessed. High concentrations of IL-4 (BALB/c-like) were represented by index values near 0, and low concentrations of IL-4 (HBA-like) were represented by index values near 1.0. The B and H values were established with three to five control mice for each group of three to six N2 mice that carried the informative recombinations we tested.

Genbank accession numbers. The accession numbers for the BALB/c and DBA/2 mRNA coding sequences for TIM-1, TIM-2 and TIM-3 are AF399827–AF399831.

Acknowledgements

We thank G. Hansen for help with preliminary airway hyperreactivity studies; V. Pete Yeung for help with mouse studies; D. Beier for input regarding *Kim1*; T. Gunn for helpful discussions; and D. Schlesinger, J. Schwartz, D. Cox, K. Frazer, M. Olivier and A. Sidow for help with map refinement and analysis. Supported by NIH-AI-24571, the Stanford University School of Medicine Medical Scholars Program, PHS grant numbers CA09302 (to J. J. M.) and CA84500 (to G. J. F.) from the National Cancer Institute and Mr. and Mrs. C. Aronstam.

Competing interests statement

The authors declare that they have no competing financial interests.

Received 15 October 2001; accepted 30 October 2001.

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